

Lamarck, Dr Steele and plagiarism

A dispute over the attribution of priority for a neo-lamarckian mechanism is premature and unseemly, to say the least, and may help make science seem ridiculous.

It is to be hoped that Dr Derek Bok, the president of Harvard University, has not been too much disconcerted by the letter of complaint that will have landed on his desk during the recent holiday asserting plagiarism by one of his university's most distinguished academics. The complaint is by Dr E.J. ("Ted") Steele of the University of Wollongong in New South Wales, and is levelled against Dr John Cairns, now a member of the Harvard School of Public Health and at one time director of the Cold Spring Harbor Laboratories, Long Island. In the belief that even university presidents deserve a break, *Nature*, which is the vehicle of Cairns's alleged misdemeanour (and which is also joined with Cairns in Steele's complaint of plagiarism) takes this early opportunity of apologizing to Bok for the trouble he will have been caused.

The circumstances are simple, but also interesting (because they bear on the mechanism of inheritance) and important (because they affect the reputation of science). In the early 1980s, Steele published two accounts of how mice whose immune systems had been challenged by certain antigens would tend to beget offspring with an innate ability to respond to the same antigen (*Proc. natn. Acad. Sci. U.S.A.* 77, 2871; 1980 and *Nature* 289, 678; 1981). The implications were recognized to be considerable. Somatic differentiation may be transferred to the germ line. Acquired characteristics can be inherited. Lamarckian inheritance is alive and well, if only in exceptional circumstances.

Given the implications, it is natural that attempts to confirm Steele's experiments should have been speedy. That was done most directly by a group that included the late Sir Peter Medawar, while Steele was an invited guest in his laboratory. Unfortunately for Steele, the attempted replication (*Nature* 290, 508; 1981) failed to support the original claim. Steele insists that the replication was not exact, and that it therefore has no bearing on his conclusions. Others hold otherwise. Steele, now at Wollongong, has understandably continued in the field.

Transgressions

Nature's alleged transgressions (and those of Cairns) stem from the publication last September (*Nature* 335, 142; 1988) of an article of which Cairns was the principal author suggesting that bacteria induced to grow in nutrient-deficient media are likely, in a somewhat lamarckian fashion, to give rise to better-adapted organisms. The interpretation of these experiments has been disputed and discussed (Scientific Correspondence, 3 November and 8 December 1988; page 123 of this issue), but Steele makes two prior points: first, Cairns and his associates, seeking explanations of their surprising observations, referred to reverse transcription as part of the mechanism whereby bacteria may be able "to exercise some choice over which mutations to accept" and, second, Cairns and his associates did not in their article refer to Steele's published work, particularly a theoretical paper that invokes reverse transcription in the generation of antibody diversity in B lymphocytes (Steele, E.C. & Pollard, J.W. *Molec. Immun.* 24, 667; 1987). The facts (Cairns *did* refer to reverse transcription, but *not* to Steele) are not in dispute. The practical question is whether that constitutes culpable plagiarism.

On the narrowest ground, that of the law, Steele is surely

mistaken. Plagiarism admittedly means copying, but plagiarists who are brought to book are people who have copied other people's texts and used them as their own. Steele cannot but be ill-advised in having broadcast his unspecific allegation of plagiarism (to the Australian press, at least, but also, in personal handwritten letters, to most members of *Nature's* London staff). What Steele means by plagiarism, of course, is not that Cairns has copied a text, but that he has stolen an idea. If, by any stretch of the imagination, that charge were true, it would be the less damaging in the courts and the more damaging in real life.

That is why Steele and the rest of us should ask what is meant by the copying of ideas. Marginally significant lamarckian inheritance is not the ground on which to look for illustrations. The heliocentric hypothesis is a better one. In retrospect, it is plain that Copernicus was the one who showed (by careful observation, and by analysis of Tycho Brahe's measurements) that the Earth goes round the Sun; Galileo, more than a century later, would not have been required to recant had he been in any real doubt about whether Copernicus was correct. But who, in retrospect, would say that Galileo was not merely a coward but a plagiarist? Apart from Steele, there can be very few.

The simple truth about this sad dispute between Steele on the one hand and Cairns (and *Nature*) on the other is that it cannot be resolved quickly, or at all, in a way that will satisfy Steele, largely because he seems not to appreciate the relative significance, in experimental science, of data and their interpretation. Copernicus is known as the originator of the heliocentric hypothesis because he was able to command a convincing body of data and show that they are most simply interpreted by supposing that the Earth goes round the Sun. Galileo was able to make the interpretation stick because he had independent observations and also a broader understanding of the laws of mechanics, winning credit for Copernicus in the process.

In Steele's case, the validity of the original data was quickly disputed. Steele's demand that an alleged plagiarism should be corrected is thus tantamount to a demand that the validity of his experimental data should be acknowledged, which would be premature. If it were otherwise, Cairns would no doubt have referred to his work, or the referees would have suggested that he should do so. As it is, Cairns has a battle on his hands persuading critics of his experiments that his data mean what they say, but he has raised the question in a manner that commands sympathetic interest.

In due course, it will become clear whether the phenomena described separately by Steele and Cairns do actually occur in nature. The possibility that one or other set of observations suggests that there are lamarckian tendencies in evolution cannot be dismissed *a priori*. Should the lamarckian case be sustained in either system, it will still be necessary to define whether reverse transcription is part of the mechanism. That will also be the stage at which it will be possible to tell who should take credit for what. In short, in a field (if it is one) that has not yet ever got a Copernicus, Steele is already talking like a Galileo. It is not unreasonable that it should take decades of hard work to bring home as radical a ship as that in whose reality Steele believes. Impatiently to fire off charges of plagiarism in these circumstances



ces is not merely inappropriate but absurd: the world knows that there are often empty quarrels over priority in science, but Steele is picking a quarrel over priority for an idea which, in most people's judgement, is literally unproven (and which may be wrong). That will not do much good for the public reputation of science. □

Monopolies die hard

British Telecom, a nationalized industry now private, seems still to hanker after old ways.

WHEN the British government decided, five years ago, to get out of the telecommunications business, one of the most powerful arguments in its favour was the calculation that a private business would perforce respond more quickly to its customers' needs than would a quasi-business run by quasi-businessmen employed as civil servants. On balance, the argument has been shown by experience to have been correct. People wanting access to telephones no longer have to wait for months or years, but only weeks or sometimes even days. Moreover, the costs of using telephones have not risen nearly as quickly as had been expected by those who remarked that the privatized nationalized industry would still retain a virtual monopoly of telephone traffic. Tariffs have been controlled by regulation, private British Telecom has made more economical use of capital than its public predecessor of the same name, telephone users have likewise been able to make the best use of their capital by choosing whether to buy or lease equipment, the volume of business has (predictably) grown more quickly than expected and, in any case, such rapacious instincts as British Telecom may have have been constrained by the generally lower tariffs offered by Mercury, its sole licensed competitor. On the face of things, privatization seems to have worked, at least in the telecommunications business. Customer power has won through.

But has it? Towards the end of last year, British Telecom published the first issue of a glossy magazine called *Telecom*, subtitled *British Telecom World*, with a cover price like that of *Nature* and stuffed with advertisements from British Telecom suppliers. Proudly, the new magazine promised to avoid being either a "gadget book" or an "equally indigestible" account of international telecommunications policy. Instead, it offered to harness British Telecom's "fund of expert knowledge and credibility" to the provision of a magazine that would be neither "a glorified brochure" nor a "marketing hand-out".

Sadly, more detailed inspection of the words gives the game away. In the old days, when British Telecom's predecessor was a government department, some British telephone users knowledgeable about practice elsewhere were used to plead that they should be given with their bills an explanation of how the imputed charges had arisen — "itemized billing" seemed to have become a customer need even while customers were still captive clients. While a public monopoly, British Telecom became skilled at dismissing these demands in a host of different ways. In the new climate in which customer demands, however whimsical, must be acknowledged to have some objective reality, the privatized company seems instead to take the view that users' yearning to know the basis on which their bills are calculated is a delusion which, if satisfied, is as likely to bring tears as a child's willingness to surrender innocence for a knowledge of grown-up life.

Thus one page of the new glossy, adorned by a tantalizing (to British users) vision of what may be an itemized telephone bill, makes two simple points. First, having planned that there should be itemized billing for all by 1995, British Telecom is now cobbling together ways of doing the job more quickly; half of Britain's telephone users should enjoy the privilege by 1990. (Oddly, the article neglects to mention that Mercury Communications offers itemized billing to all its customers already.) Second, which is where the old nanny comes in, crusading

Telecom disinters the old excuse that the provision of the right to know how a telephone bill is calculated "would distract valuable engineering resources" from the task of modernizing Britain's telephone system. Much in the spirit of "... they know not what they do", *Telecom* remarks that large users are not equipped to handle bulky bills, while some spouses would wish guiltily to conceal their telephone usage from their partners.

The sad moral in this gloomy tale is that privatization by itself does not change public monopolies into private companies, but that time is also necessary if an organization's facetious scorn for the private persons who provide its revenue is to melt away. Mercifully, for the customers, British Telecom has a geographically omnipresent competitor. The British government, which is this year planning to privatize both the nationalized electricity and water industries, should even at this late stage give some thought to the circumstance that monopolistic complacency will there be more easily sustained. □

Hope before reality

The British government's latest view of higher education hangs on assumptions still unproven.

BRITISH academics should not let their emotions get the better of their judgement when reading reports of Mr Kenneth Baker's speech at Lancaster last week (see page 105). After a decade of enforced contraction of the British university system, it is bound to seem mystifying that the Secretary of State for Education and Science should be talking of a quite different world in which the proportion of young people in higher education has doubled. People are bound to ask what perversity can explain why the university system should first contract so that it can grow to a size sufficient to meet modern needs. Before they accuse Baker of inconsistency, or worse, they should allow that he was talking last week of a university system quite different from that to which his predecessors took an axe in 1980. The question is not whether Baker is being inconsistent but whether the system of which he now speaks is more substantial than a mirage.

The objective, or hope, is that there will emerge from present academic institutions a system of higher education that is more diverse than at present, thus catering more adequately for the demands of the supposed market in higher education, but whose growth (at least) will be sustained by other than public sources of funds. Baker apparently has in mind the fees paid by eager students and the contributions that successful industries will be impelled to make in academic institutions which are essential to their own well-being. Baker likened his vision of British higher education to that of higher education now in the United States. British academics and universities might even embrace that vision if they believed that it would come true. The truth, as always, is more complicated. Even the analogy with the United States is misleading. The University of California system, comparable in size with the British university system and qualitatively enviable, is supported by the state of California.

Many other state systems are almost in the same league. Moreover, while many of the private universities in the United States are outstanding (and outstandingly successful) academic institutions, they enjoy the benefit of large endowments accumulated over a century of prosperity — not to mention the continuing generosity of their alumni. Yet experience in the United States has shown that industry is not the endless source of funds that it might be, even with much more generous tax incentives than the British government has put in place. Nor does it make sense that higher education, which is in the best sense subversive of present contentments, should become over-dependent on self-interested industry. Baker's hope that British higher education can prosper in an entirely free market turns on the assumption that Britain is certain to reach that prosperous condition his financial colleagues in the government are fond of talking of, but that puts the cart before the horse. □

UNESCO chemical arms conference opens in Paris

- Soviets ready to destroy chemical weapons
- Poison gas the poor man's nuclear deterrent

Paris

THE French President, Francois Mitterrand, opening a marathon five-day conference on chemical weapons in Paris on Saturday, reminded the 143 national delegates that "this is not a tribunal". Recent accusations in the United States that Libya is building a massive chemical weapons plant, compounded by its shooting down of two Libyan fighter planes last week, had put Arab states on the defensive and created a cloud of hostility even before the debates had begun.

As some nations only agreed to attend if there would be no finger-pointing, Mitterrand, as host to the conference, is believed to have asked the United States to calm down when he met the Secretary of State, George Shultz, on Friday.

But what was to have been a diplomatic gesture to reaffirm an existing ban on the use of chemical weapons had already, by Saturday evening, acquired political muscle. In a surprise gesture, the Soviet Foreign Minister, Eduard Shevardnadze, raised the stakes, agreeing to halt production of chemical weapons and to destroy existing stocks, even before a formal treaty has been signed.

The conference is the result of initiatives first by the US President, Ronald Reagan, then by Mitterrand, proposed at the tribune of the United Nations General Assembly last September. The original aim was to reinforce the 1925 Geneva Protocol, drawn up to prevent a recurrence of the horrendous casualties of chemical weapons during the First World War. By 1975, when the United States ratified its signature, 97 states had signed the protocol.

A further 12 have joined since then. But the protocol only outlaws the use of chemical arms, not their manufacture or stockpiling. Even so, there have been flagrant contraventions of the protocol, most recently when Iraq used chemical weapons against Iran and its own Kurdish population.

According to estimates in the United States, as many as 22 nations are suspected of possessing chemical weapons, but only two — the United States and the Soviet Union — officially admit having stocks. Against what appears as an increasingly cynical disrespect for the Geneva Protocol, Mitterrand hopes the conference will go further, leading to the worldwide ban on the "use, manufacture or stockpiling" of chemical weapons. According

to the French Foreign Minister, Roland Dumas, a draft agreement has already been drawn up which, he hopes, all national delegations will sign on Wednesday when the conference ends.

Such an agreement could give a welcome push to the 40-member United Nations disarmament committee which has been battling in Geneva for 18 years to ban the production and stockpiling of chemical weapons. The committee has met with two major stumbling blocks.

First, non-industrialized countries see chemical weapons as a "poor man's nuclear bomb". Their presumed deterrent effect is particularly valued by countries such as Syria, faced with the nuclear capability of neighbouring Israel. Indeed, Iraq's use of chemical weapons is thought to have played a role in ending its war with Iran. But, say critics, this surely shows that possession of chemical weapons is not a deterrent to their use in war — an argument that has nevertheless been defended by the United States, the Soviet Union and by France who have only agreed not to use chemical arms first in a conflict.

Second, it is difficult to verify that nations are not secretly producing chemical weapons. Most poison gases are produced by combining chemicals that have legitimate industrial uses. Mustard gas, for example, is made by mixing hydrochloric acid with thiodiglycol, an agent used to improve ink-flow in ballpoint pens. Modern sophisticated chemical manufacture makes it easy to divert a legitimate plant to the production of weapons gases and to conceal the process in time for an inspection. Furthermore, the minute examination which could reveal sinister uses of a chemical plant would also leave nations vulnerable to industrial espionage by international investigators.

On Sunday, the conference was still dominated by mud-slinging between industrialized and developing countries. But the Soviet move will challenge the United States — who have recently raised the temperature by designing a new generation of 'binary' chemical bombs — to follow suit. This is unlikely unless Arab nations change their stance. The remaining three days are therefore likely to be used to explore ways of softening entrenched attitudes. More than 75 foreign ministers are in Paris for the talks, and they are making the most of the opportunity for informal meetings. **Peter Coles**

Backing for Libya

PRESIDENT Nicolas Ceaucescu of Romania, who frequently presents himself on the world stage as an advocate of disarmament, now argues that less-developed nations should be allowed to have chemical weapons — until such time as the superpowers destroy both their chemical and nuclear arsenals. Last week, he said that Romania would not commit itself to any international agreement that dealt with chemical weapons in isolation (see left). "Ultimately, even chemical arms can be a deterrent for those who have no nuclear arms. . . It is difficult to believe that many peoples will want to remain unarmed, to be in a position — as is the case of Libya now — to be told 'unless you obey the will of one country or another we come and bombard you'."

During the past year, Romania has antagonized other countries with actions ranging from destruction of villages (flooding Hungary with refugees), chemical fumes pouring into Bulgaria and Ukraine, a scandal over hazardous wastes imported into the Black Sea port of Sulina, and the alleged diversion of heavy water intended for a Romanian nuclear power plant to Israel. If, as many conclude, Ceaucescu's remarks are indirect confirmation that the disputed Libyan chemical plant at Rabta is not simply a civilian facility, then two questions arise: how did he know this; and why reveal it? **V.R.**

Benveniste cont. . .

DR Jacques Benveniste, whose work on the alleged 'molecular memory' of water put him at the centre of a major controversy last year (see *Nature* 333, 816, 334, 287 and 335, 759; 1988), has been challenged by the popular magazine *Science et Vie* to reproduce his experiments in a 'controlled' context stipulated by the magazine. But he has refused, saying his research is not accountable to people "not even qualified for the job of porter" in his laboratory. **P.C.**

Numbers game

BUSINESS and finance courses are attracting undergraduate students to universities in droves, while numbers in some science disciplines are increasing only slowly or decreasing, according to statistics published in Britain this week by the Universities Statistical Record. Between 1985–86 and 1987–88, the number of undergraduates studying business and finance increased by 16 per cent from 9,300 to 10,800. The greatest increase among the science disciplines was 5.6 per cent, to 16,800, in the biological sciences. There were smaller increases in those studying engineering, technology and mathematics, and decreases in physical sciences, in medicine and dentistry and in veterinary science and agriculture. The number of postgraduate students increased in all sciences except engineering and technology. **C.McG.**

President-elect Bush gets some free advice on science policy

Washington

IN AN unprecedented move, the National Academy of Sciences, the National Academy of Engineering and the Institute of Medicine have decided to offer some advice to the new US administration about to occupy the White House, rather than take the more traditional course of waiting until their advice is sought.

The three academies are presenting President-elect George Bush's transition team with four white papers (policy documents) on space policy, AIDS, global environmental change and science advice in the White House. Frank Press, president of the National Academy of Sciences, acknowledges that the transition team is flooded with such documents, but insists that the scientific community has a unique point of view that deserves to be heard.

According to Press, the topics chosen are not the only important ones facing the new administration, but rather reflect projects that have recently been addressed by one or more of the academies. He argues that scientific expertise must be brought to bear on a wide variety of domestic and

international issues, and that Bush will need a science adviser with a staff prepared to call attention to topics in crucial need of attention. Otherwise, critical issues may not receive the kind of timely attention they deserve. As an example, Press points to the recent report from the special committee to advise the president on high-temperature superconductivity (see page 105). Although the report is excellent, he says, it, would have had more impact had it been produced a year ago.

In a campaign speech in October, Bush promised to raise the status of the science adviser to assistant to the president. The white paper urges him to honour that promise. By raising the status of the position, it will be easier to recruit a top scientist to take the job.

One of the first big science decisions facing the new administration will be what to do with the planned space station. Congress has ordered that \$515 million for the project be held up until May when Bush must announce his plans.

Although the white paper on space

policy does not take a position on whether or not the station is a good idea, it urges Bush to maintain a core budget for NASA (National Aeronautics and Space Administration) separate from the space station for a base programme that will "ensure US competence in all space activities". The core budget would include money for launch systems, space science and remote Earth sensing, and support for new technology. Supplemental budgets would cover large projects such as the space station or manned exploration of the Moon or Mars.

The new president will also inherit a pressing need to take steps against the growing problem of AIDS, according to the academies. The disease is expected to cause 200,000 deaths during Bush's four-year term of office, and there is still no effective therapy. The AIDS white paper spells out specific actions intended to limit the spread of human immunodeficiency virus (HIV), including making effective use of the newly legislated National Commission on AIDS; preventing discrimination against HIV carriers; instituting "aggressive and unambiguous educational programs" as well as improved surveillance and reporting measures. The administration will also have to address the serious question of how to pay for the increasing costs associated with treating people with AIDS.

Although the AIDS report acknowledges the need for rapid development of new therapeutic agents, it warns against the use of new Food and Drug Administration regulations that permit the distribution of drugs whose effectiveness is still unknown. These new procedures could interfere with the ability to execute "conclusive clinical trials", making it hard to determine whether new drugs actually work as intended.

Finally, the academies suggest that the issue of global environment "must be made more prominent in the scientific, political and foreign policy agendas of the United States". Many federally sponsored activities affect the global environment, and these must be coordinated. The white paper urges the new administration to consider immediate policy changes, such as a reappraisal of nuclear and solar energy and increasing use of the "cleanest" fossil fuels, as strategies to combat global warming. Preservation of rain forests, reduced chlorofluorocarbon emissions and better monitoring of the effects of global warming should all be high on the new president's agenda.

Although he would not be specific, Press says the white papers have been well received by "very senior people" in the new administration. So far, however, the Bush transition team has not announced who will take the science adviser's job, or whether that job will be different from the one that now exists.

Joseph Palca

Administration's 1990 budget proposal

THE final budget proposal of the Reagan Administration was sent to Congress on Monday this week as *Nature* was going to press. As in previous years the President's budget is just a starting point for the budget, as there will undoubtedly be changes both from the new Bush administration and Congress.

The Energy Department total includes a separate line item of \$250 million for the Superconducting Super Collider which the administration proposes to build in Waxahachie, Texas. The apparent decline in the budgets for the Centers for Disease Control and the National Institutes of Health are explained by the fact that AIDS research now appears as a separate line item for AIDS amounting to \$1,600 million. In fact, the budget includes a 6.6 per cent increase for basic research budgets. There is also \$100 million set aside for the human genome project. The NASA budget includes \$2,100 million for the manned Space Station. There is also \$5,900 million proposed for the Strategic Defense Initiative.

	1989 (estimate)	1990 (proposed)
	(millions of dollars)	
Department of Agriculture		
Agricultural Research Service	579	600
Cooperative State Research Service	341	295
Department of Commerce		
National Oceanographic and	1,270	1,078
Atmospheric Administration		
National Institute of Standards and Technology	159	156
Department of Energy		
Atomic Energy	8,100	9,027
Defense Activities		
General Science and Research Activities	922	1,169
Biological and Environmental Research	269	271
Department of Health and Human Services		
Centers for Disease Control	979	574
National Institutes of Health	7,147	6,777
Department of Interior		
US Geological Survey	452	453
Environmental Protection Agency	3,830	3,043
National Aeronautics and Space Administration (NASA)	10,969	13,148
National Science Foundation	1,885	2,149

British government reviews its plans for student loans

London

THE British government is being forced to rethink its plan for the introduction of loans to students in higher education to replace the maintenance grant they receive at present. The Department of Education and Science was warned last week that the administrative costs of the proposed system will outweigh the savings. Banks and building societies, which the government planned should administer the scheme, are now reluctant to commit themselves without reassurance that the government will meet some of these costs.

In a paper written for the Department

of Education, Nicholas Barr of the London School of Economics says that the annual administration costs of the scheme would be £100 per student, resulting in a total annual cost within 15–20 years of £250 million. The government's policy paper on loans, published last November, fails to take account of these costs and foresees annual savings of £230 million once the scheme is established. An alternative method of repayment of loans is now under consideration. This method, which involves an addition to graduates' national insurance contributions, would be administratively very simple and cheap, says Barr. And because evasion of repayment would be more difficult, resulting losses would be no more than 10 per cent of the total as opposed to the predicted 25 per cent losses under the original plan.

Outlining his vision for higher education in the next 25 years, the Secretary of State, Kenneth Baker, said at a conference last week that the British system should be expanded along the lines of that in the United States (see page 102). The present British system in which higher education is dominated by the role of the state would remain for the foreseeable future, he said. But continued dependence on public funds would hinder moves towards greater autonomy and diversity. Baker's vision of an expanded higher education system includes an influx of older students, women and members of ethnic minorities.

● Overseas students in British universities face steep increases in fees after the Committee of Vice-Chancellors and Principals (CVCP) raised the prices for next year's courses by 10 per cent. At a time when countries such as Australia and Canada are trying hard to attract a bigger share of the world market in students, Britain is risking losing more overseas students, according to Andrew Mascheter, director of the UK Council for Overseas Student Affairs.

The CVCP this year took over the role of pricing courses from the University Grants Committee. And instead of setting a minimum price, it set what it calls a form of "recommended retail price", allowing universities to set higher or lower prices as they choose. Although it is unlikely that universities will lower prices in the near future, price-cutting could take place as competition between universities for overseas students becomes more aggressive. Recruiters for overseas students at the universities are worried that this might happen and say that if prices drop then standards too will drop.

Christine McGourty

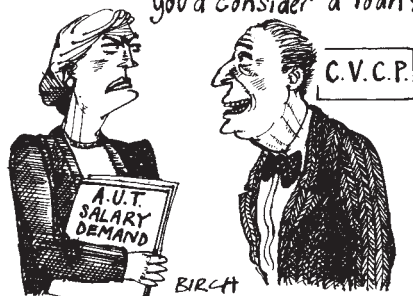
Clash over salaries

London

THE dispute over a salary increase for academic staff in British universities turned into a crisis this week as staff began a boycott of the examination process to put pressure on the vice-chancellors to make an offer for last year. Discussions between the Association of University Teachers (AUT) and the Committee of Vice-Chancellors and Principals (CVCP) have now dragged on for a year.

Both parties agree that a salary increase

Mr. Baker wonders if you'd consider a loan?



is necessary, but the CVCP says there is no money to finance it; any offer would have to be so low as only to inflame the situation, and anything else would risk bankrupting some universities, it says. The AUT insists that a modest increase is affordable. Meanwhile, the CVCP is trying to persuade the government to provide extra cash for a salary increase for 1989–90. It says that although pay has gone up by about 8 per cent a year over the past three years, it stands at less than 80 per cent of its value ten years ago.

Under the terms of the boycott, the 30,000 members of the AUT are expected not to set or mark examination papers or to invigilate exams. The CVCP has warned staff that such action constitutes a serious breach of contract. Christine McGourty

Surprise candidate

ACADEMICIAN Andrei Sakharov has been nominated as a candidate in the forthcoming Soviet parliamentary elections — the first to be held on a multicandidate basis. His nomination was approved at a meeting at the Pavlov Institute of Physiology of the Soviet Academy of Sciences in Leningrad on 5 January. Sakharov was not present at the meeting but his supporters say that "his opinions and civic standing are well enough known for him to be regarded as capable of defending the cause of *perestroika* and renewal of Soviet society". V.R.

New Wolfson awards

UNDER a novel awards scheme, the Wolfson Foundation is making grants not tied to specific research projects; instead, they are made to researchers so that they can employ additional staff and buy equipment. Eighty-five grants, worth £6 million, were announced last week. A further £4 million will be spent in 1991. C.McG.

Super consortia plans

Washington

A special advisory committee formed to make recommendations to the White House on how best to realize the commercial possibilities of the new class of high-temperature superconductors last week released its conclusions. The so-called 'wise men's' committee suggests creating about half a dozen Superconductivity Consortia along the lines of the NSF's Science and Technology Centers, bringing together universities, industry and government laboratories. The committee, chaired by Ralph Gomory of IBM, also suggests there is extra research capacity that could help the commercialization effort. J.P.

Synchrotron go-ahead

THE European Synchrotron Radiation Facility (ESRF) has finally been given the green light, 14 years after the European Science Foundation set up its first working party to investigate its feasibility. Representatives of the 11 participating countries met in Paris in December to sign the documents approving the project. ESRF, which will cost FF2,600 million (\$434 million), will take 6 years to build and will be the world's most powerful synchrotron radiation facility. The first beam line will be ready on the Grenoble site in 1994 and the full 30 beam lines by 1998. P.C.

Nature movements

ALUN Anderson, Washington Editor, will be based in the Tokyo office from 18 January to 18 March. Joseph Palca, Washington News Editor, will act in his stead in the interval. David Swinbanks, Tokyo correspondent, will be based in the Washington office during the same period. John Maddox, Editor, will be based in Washington from 1 to 28 February. □

Another vaccine enters the fray

Tokyo

JAPAN'S Chemo-Sero-Therapeutic Research Institute (Kaketsuken) plans to export a newly developed recombinant hepatitis-B vaccine to South-East Asia and China where the virus is one of the main causes of cancer. The vaccine will compete with similar products made by US and European pharmaceutical companies. But the Japanese vaccine is unlikely to make significant inroads outside Japan until it is much cheaper.

The institute's plans to export the vaccine, will be finalized within the next six months, according to Satoru Tsutsui, manager and patent attorney for the institute's Kikuchi Laboratory in Kumamoto in Kyushu, Japan's southern island. Many problems, however, remain to be resolved over patent rights for the new vaccine.

Hepatitis B is a leading cause of cancer in the Far East. In Japan, several million people are carriers of the virus and each year more than 100,000 people are infected, about half of whom will develop liver cancer. In China and South-East Asia, at least one in ten are carriers.

Kaketsuken's vaccine was developed in collaboration with Professor Kenichi Matsubara of the Institute of Molecular and Cellular Biology of Osaka University with the support of an ¥830-million (\$7 million) grant awarded in 1984 by the Research and Development Corporation of Japan, an affiliate of the Science and Technology Agency (STA).

The vaccine is made of viral proteins mass-produced in a yeast host, a technique also used by the pharmaceutical companies Merck and SmithKline. Japan's Shionogi and Banyu pharmaceutical companies have received permission to import Merck's vaccine to Japan, and several other Japanese pharmaceutical companies are making similar arrangements. But Kaketsuken is the only Japanese organization which has developed and is producing and marketing its own recombinant vaccine.

STA and the institute have applied in Japan, the United States and Europe for patents for the recombinant plasmid used in production of the vaccine, and a US patent was recently issued. But Tsutsui admits that there is "probably" overlap with patents filed by other companies.

The market at stake is enormous. In Japan alone, Tsutsui estimates that there is a need for about a million doses of the vaccine in the first year of production. This will tail off after a few years to about 50,000 doses a year for newborn babies once most adults are immunized.

David Swinbanks

Greenpeace's running battle with France now in Antarctica

Sydney

THE French government has locked horns again with the environmental group Greenpeace, this time over alleged violations of the Antarctic Treaty. The Greenpeace ship, MV *Gondwana*, arrived in the Antarctic last Friday to protest against the construction of a runway at the French Antarctic base, Dumont d'Urville. Early on Saturday morning, construction site personnel attempted forcibly to remove blockades set up by the protesters. Peter Wilkinson, expedition coordinator, said

that "the French construction men (were) not much better than hooligans looking for a fight". Work on the airstrip has ceased while the construction crew await orders from Paris.

Greenpeace are protesting at the fact that, in the past six years, five islands have been flattened with explosives and the channels between the islands filled in. This has resulted in the blocking of the traditional migratory path of a colony of emperor penguins and the destruction of the breeding grounds of the Adélie penguin. According to a spokesperson for Greenpeace, Michael Bland, this constitutes a violation of the Antarctic Treaty.

Greenpeace claims that the runway is being built to provide the infrastructure for the future development of mineral exploitation. The *Gondwana*'s protest coincides with a campaign to discourage Australia from ratifying an agreement allowing oil and mineral exploitation in the Antarctic. Greenpeace has accused Australia of complicity with the French.

The French expedition is using Hobart in Tasmania as a staging post for delivery of equipment and materials to Dumont d'Urville. "We have told the Australian government that this equipment is not for scientific use but they have chosen not to protest to the French", Bland said. The Federal Department of Environment in Canberra declined to comment.

Tania Ewing

IMAGE
UNAVAILABLE
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REASONS

French construction workers keeping Greenpeace supporters out of their survival hut (AP).

Shake-up for UK electronics industry

London

THE latest move in the re-shaping of the European electronics industry could result in the take-over of Britain's largest electronics group, the General Electric Company (GEC), by an international consortium led by Plessey, the country's second largest electronics company. A firm bid, which will need to be in the region of £7,000 million, has not been made and the members of the consortium are not yet known, but it is thought likely that it will include the British companies Plessey and STC, as well as Thomson, the state-owned French company, and General Electric and AT&T of the United States.

The existence of the consortium was announced this week by Lazard Brothers, the merchant bank which is advising Metsun Limited, the company set up to make an offer on behalf of the consortium; the same bank is advising Plessey on its defence against the joint take-over bid by GEC and the West German company, Siemens. The announcement was greeted with speculation that it was an attempt to

force a referral of the £1,700 million GEC-Siemens bid for Plessey to the Monopolies and Mergers Commission. GEC and Plessey are among the biggest suppliers to the Ministry of Defence, which is keen to maintain a competitive procurement policy. It is not clear how those behind either bid will assure the ministry that competition will not be lost nor how the diverse interests of all the companies thought to be behind the latest take-over bid would be combined.

British electronics companies are constantly criticized for their reluctance to invest sufficiently in research. The managing director of GEC, Lord Weinstock, has built up a £1,500 million cash mountain while excellent research in GEC laboratories lacks the investment needed to turn that research into marketable products. But it is feared that if foreign interests were to dominate a consortium that controlled the company, then much of the defence research, which is the driving force of Britain's electronics industry, could go abroad. Christine McGourty

US team returns with insights into Armenian earthquake

Washington

ACCORDING to a team of US seismologists and engineers that has just returned from the Soviet Union, the extraordinarily high death toll in the Armenian earthquake on 7 December was due in large part to poor assembly of prefabricated building parts, compounded by simple bad luck. Whereas most large earthquakes produce injuries and fatalities at a ratio of three or four to one, the numbers in this catastrophe were reversed.

Loring Wyllie, a structural engineer and co-leader of the 18-member team, said that although the town of Leninakan had suffered almost total devastation, older buildings and a few of the newer types had fared reasonably well. The most common apartment building style was a 9-storey frame construction, in which factory-made walls and floor are attached to a system of beams and columns. Out of 50 such buildings, fewer than 12 remained standing, and all were severely damaged. But about a dozen other buildings of a panel construction (in which the walls and floors are slotted together to form their own support), suffered much less damage.

Although analysis from the rubble was difficult, the engineers believed that the greatest fault with the frame buildings was that most of the stress is put on a fairly small number of connection points, and these connections were often poorly assembled. In the panel-construction buildings, by contrast, the load is spread more widely, and the joints can tolerate some movement before collapsing.

The faults of the frame building were magnified because the earthquake actually consisted of two strong shocks, with a tremor of Richter magnitude 5.8 coming 4 minutes after the initial 6.8 magnitude event. From the pattern of fatality in the wrecked buildings, the investigators concluded that the first shock had not demolished many of the apartment blocks, and that in the intervening 4 minutes residents had tried to escape, but had reached only the lower floors before the buildings collapsed on top of them. When the joints in the frame buildings failed, the floors collapsed vertically, leaving almost no spaces where people might have survived.

Fred Krimgold, of the College of Architecture and Urban Studies at Virginia Polytechnic Institute, said it was difficult to say whether the buildings were adequately designed, and might have done better had they been more carefully assembled. The basic designs came from Moscow, and had been modified for the local seismic conditions, but there seemed to be frequent discrepancies between the architectural drawings given to the team

and the construction of the buildings themselves. According to Krimgold, the quality of the prefabricated parts seemed to be adequate, but there were many cases where parts had not fit together properly and joints had been improvised.

Seismologists with the US team, led by John Filson of the US Geological Survey, monitored the area for aftershocks and mapped out the fault region. A surface rupture of the Earth was found a few kilometres south of the epicentre, and about half way between the towns of Leninakan and Kirovakan. One of the puzzles confronting the seismologists was that damage in Kirovakan was relatively slight, although it is closer to the fault than Leninakan and has identical buildings. One small section of Kirovakan had been severely damaged, and local residents told the seismologists that the area was a

drained marsh. This led Filson to suspect that local geological conditions created seismic resonances in some areas, amplifying the tremors. A similar phenomenon is thought to have occurred in the Mexico City earthquake of 1985.

About 24,000 dead have now been found, but Krimgold suspects that a total fatality rate of around 60,000 is likely. Many had died of exposure in the days after the earthquake, and to add to the uncertainty there were rumours that as many as 100,000 refugees from the disturbances in Azerbaijan were in the area, of whom an unknown number might have perished.

Members of the team were impressed with the cooperation they were given by Soviet and Armenian officials, and by the candour of their discussions. Krimgold believes that the Soviets are anxious to learn from the disaster, and are already making moves to discard unsafe building designs and to revise building practices, perhaps by using fewer prefabricated parts and pouring more concrete on-site.

David Lindley

Japan loses its most illustrious biologist

Tokyo

JAPAN'S Emperor, who died last Saturday, was a dedicated natural scientist who made several significant contributions to marine biology and botany. Emperor Hirohito's interest in marine biology began at an early age during visits to the imperial villa at Numazu on the coast of Suruga Bay south of Tokyo. In 1918, when still the teenaged crown prince, Hirohito discovered a new rare species of Pacific prawn, named *Sympasiphaea imperialis* after him, on the

shore behind the villa.

Ten years later, after ascending the throne, he established a personal research institute in the grounds of the palace in Tokyo. Barring official engagements, the emperor spent every Monday, Thursday and Saturday studying marine organisms, fungi and plants collected from the grounds of the palace and from various imperial villas dotted around the country.

Hirohito has numerous publications to his name, including a book on the hydrozoans of Sagami Bay, one of a series of volumes on the bay's fauna, which was published in August of last year shortly before he fell ill. His research has won him membership of the Royal Society of London and honorary memberships of the Linnean Society and the Zoological Society of London.

In 1975 he visited Woods Hole Oceanographic Institution, the Smithsonian Natural History Museum, and the Scripps Institution of Oceanography in the United States. It is said that he had to be persuaded to leave after spending an all-too-short hour with Scripps scientists Robert Hessler and William Newman examining hydrozoans.

The emperor has passed on his enthusiasm for biology to his children and grandchildren. The new Emperor Akihito is a keen marine biologist who studies goby fish. Akihito's brother Prince Hitachi is a cancer researcher, and the new emperor's second son Prince Aya, who is currently at the University of Oxford, is a specialist in the taxonomy of catfish. Biological research seems destined to continue at the palace.

David Swinbanks

IMAGE
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Japan's most illustrious biologist (AP).

More money needed for human genome mapping project

Bethesda

THE Reagan Administration is seeking an almost fourfold increase in spending for 1990 on a project to map and sequence the human genome, up from the \$27.6 million the National Institutes of Health (NIH) will spend on genome activities this year. If this huge increase is approved by Congress it will mean much extra responsibility for the newly christened Program Advisory Committee on the Human Genome, chaired by Norton Zinder of Rockefeller University. NIH are counting on the committee for guidance as they work to sequence the 3,000 million bases that make up the human genome.

NIH director James Wyngaarden gave his agency an active role in the genome project last year when he established NIH's Office of Human Genome Activities and chose as its director James Watson, director of the Cold Spring Harbor Laboratory. The choice of Watson will make it hard for Congress or anyone else to ignore the project. For his part, Watson sees the project as important to the scientific community, not only because of its ultimate goal, but also because it will generate new technology along the way.

Watson's office exists within the office of the director, and does not have legal authority to make grants itself. For the time being it will coordinate its spending choices with the National Institute of General Medical Sciences.

Unlike some advisory groups, Zinder's committee is being urged to take a hands-on approach as NIH formulate their plans. It will also make up half the membership of a joint committee with the Department of Energy that will coordinate the two agencies' activities as prescribed by a memorandum of understanding signed last autumn.

The National Science Foundation and the Department of Agriculture will also support human genome research, although the Department of Agriculture's contribution will be small, at less than a million dollars. The Howard Hughes Medical Institute will also play an important role in the project, with its support of genetic databases and mapping efforts.

At the advisory committee's first meeting last week, four working groups were set up: one on training chaired by Joseph Goldstein of the University of Texas Southwestern Medical Center in Dallas; one on centres that will work on the pro-

ject co-chaired by Phillip Sharp of the Massachusetts Institute of Technology in Cambridge and Maynard Olson, Washington University School of Medicine, St Louis; another on ethics chaired by Nancy Wexler of Columbia University in New York; and the fourth on databases chaired by David Botstein of Genentech, Inc. in San Francisco.

Olson, who developed the yeast artificial chromosome technology which will probably be used to construct a physical map of the genome, urged the group to pay attention to the issue of resource sharing. Olson says it is difficult to determine how much time individual researchers should be expected to spend in assembling requested resources, even though in principle he believes that reagents developed

using public money should be freely available.

While the advisory group is putting together its recommendations, Watson plans to travel around the country, visiting laboratories and talking to university officials. He says that new construction money is needed from Congress to convince universities to act as hosts for genome centres.

Watson believes the entire project can be completed in 15–20 years. A first step will be to upgrade databases containing gene sequences from other organisms, particularly the fruitfly *Drosophila*, so that human sequence information can be compared to known gene sequences as it is obtained.

A complete physical map of the genome should be complete in 5–7 years, Watson says, although he adds that "Maynard [Olson] looks pained when I say five years because he knows I am counting on him".

Joseph Palca

FDA sceptical about link between breast cancer and the pill

Washington

THE US Food and Drug Administration (FDA) last week decided not to add a warning to the labels on birth-control pills after examining new evidence said to link use of the pill to breast cancer. An FDA advisory panel composed of gynaecologists and epidemiologists ruled that data from three independent studies were inconclusive and recommended that more comprehensive studies be conducted before alarming the 13.2 million women in the United States who take the pills.

Earlier studies have uniformly shown that the pill does not increase risk of breast cancer and may even help lower the risk for cancer of the ovaries and uterine lining. The contradictory new data come from a paper by Samuel Shapiro of Boston University to be published in the *American Journal of Epidemiology* next month, an ongoing study sponsored by the British Royal College of General Practitioners, and a reanalysis of the \$10 million Cancer and Steroid Hormone (CASH) study sponsored by the US National Institute of Child Health and Human Development.

All three of the studies found the rate of breast cancer increases among young women and women who have been taking birth-control pills for less than ten years. Shapiro's study of 400 women showed that women who have used the pills for less than 10 years had twice the incidence of breast cancer by age 45 than women who did not. The Royal College study led by Clifford Kay, based on data reported by individual physicians on 46,000 women, found no overall increase in cancer among users of the pills, but did find an increase

among 30–34-year olds. The reanalysis of the CASH study by Bruce Studer based on 5,600 women shows that childless women with an early menarche who take the pills for more than seven years increase their risk of cancer by 2.7 times.

But each of the studies has technical flaws, and the FDA panel is sceptical of their contradiction of earlier studies which have shown the pills to be safe. The consensus at the FDA meeting was that such surprising increases in breast cancer among younger women could indicate that birth-control pills promote the development of cancer, but do not initiate it. According to Malcolm Potts of Family Health International, if the promoter theory is correct, studies would show increases in the number of young women developing cancer, and drops in the number of post-menopausal women, because women who were already destined to develop cancer would be doing so at an earlier age. The first cohort of women to take the pills are now in their late 40s, so the predicted dip in the number of cases would not yet have been seen.

A study of 2,000 women which will test this theory has just been launched by the US National Cancer Institute but the results may not be in until the middle of the next decade. The ongoing British study will not receive government funding next year and may be ended.

Health watchdog Sidney Wolfe of the Public Citizen Health Research Group intends to file a petition to require the FDA to reverse its decision not to relabel the pills, and if the agency does not he will bring suit.

Carol Ezzell

● In a News item entitled "West Germany voices objections to European genome project" (*Nature* 336, 416; 1 December 1988), the potential European market for DNA probes should have been given as 1,000–2,000 million ECU a year. □

Partnership research success

Kumamoto

THE first of several new research institutes designed to promote collaborative research between Japanese universities and industry has opened in Kumamoto in the southern island of Kyushu.

The Cooperative Research Center of Kumamoto University is in a technoresearch park that forms the nucleus of the Kumamoto 'technopolis'. Technopolises are being established in dozens of communities around Japan under the authorization of the Ministry of International Trade and Industry (MITI) in an attempt to promote research and development activities and high-technology industry in local regions.

The cooperative research centre, established by the Ministry of Education, Science and Culture (MESC), accepts contracts for joint research between Kumamoto University and industry. Twenty-one research projects are under way on subjects ranging from development of plasma chemical vapour deposition techniques for making thin films of high-temperature superconductors (a collaborative project with Dojin Chemical Company and Denki Kagaku Kogyo) to the design of skirts for hovercraft. MESC is establishing seven other cooperative research centres around the country and more are planned in fiscal year 1989.

The contracts are small — a few thousand to a few tens of thousands of dollars a year — but the companies also provide researchers and equipment. Patents arising from the research are held jointly by industry and the central government. Such collaborative industry/university research has increased dramatically since 1983 when MESC introduced a new system to encourage joint research. In 1988 about 400 projects were supported with funds of ¥2,600 million (about \$20 million).

Donations from private industry to national universities have also increased substantially to about ¥25,000 million (\$200 million) in 1986. But the prime motive of companies for making such donations is to obtain a steady supply of top graduate students.

Several big high-technology facilities are already established in the area. NEC has the world's largest integrated chip factory, the Chemo-Sero-Therapeutic Institute has established a new research laboratory nearby (see page 106), and Tokai University built a space information centre next to the park. Kumamoto government officials are confident that many more high-technology companies and research institutes will soon come to the area.

David Swinbanks

Nature's East-West exchange

The following have endorsed *Nature's* East-West exchange described in last week's issue (page 1). Those appearing on this list are those whom it was possible to reach by telephone or telefax over the recent holiday; the absence of names has no significance. Those wishing to participate in the exchange scheme should write to the Editor of *Nature*, not to those whose names appear below, who may not be in a position to help directly.

Professor Eduardo Amaldi, president, Accademia Nazionale dei Lincei, Rome, Italy. Professor Bruce Alberts, Department of Biochemistry and Biophysics, University of California, San Francisco, USA. Professor Eric Ash, rector, Imperial College, London, UK. Professor John Cairns, Department of Cancer Biology, Harvard School of Public Health, Cambridge, Massachusetts, USA. Professor C Thomas Caskey, Department of Medicine, Baylor College of Medicine, Houston, Texas, USA. Professor Umberto Colombo, chairman, ENEA (Italian National Agency for Nuclear and Alternative Energy Sources), Rome, Italy. Dr George A Cowan, president, Santa Fe Institute, Santa Fe, New Mexico, USA. Dr Nina Federoff, Carnegie Institution, Baltimore, Maryland, USA. Dr Donald S Fredrickson, former director, National Institutes of Health, USA. Professor emeritus S. Fukui, Kyoto University, Japan. Dr Koji Fushimi, past president of Science Council of Japan. Dr Robert C Gallo, chief of Laboratory of Tumor Cell Biology, National Cancer Institute, USA. Dr Richard Garwin, IBM, Yorktown Heights, New York, USA. Professor Vitaly Ginzburg, P N Lebedev Institute, Academy of Sciences of the USSR. Professor Stephen Jay Gould, Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts, USA. Professor D Hempel, director, Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, West Germany. Professor Ira Herskowitz, Department of Biochemistry and Biophysics, University of California, San Francisco, USA. Sir Alan Hodgkin,

past president of Royal Society, London, UK. Professor David Hogness, Department of Biochemistry, Stanford University, Palo Alto, California, USA. Sir Andrew Huxley, past president of Royal Society, London, master of Trinity College, Cambridge, UK. Professor Thomas H Jukes, University of California, Berkeley, USA. Professor John W Kappler, University of Colorado Health Sciences Center, Denver, USA. Professor John Knill, chairman, Natural Environment Research Council, UK. Dr Joshua Lederberg, president, Rockefeller University, New York, USA. Professor Victor McKusick, Department of Medical Genetics, Johns Hopkins University, Baltimore, Maryland, USA. Professor Philippa Marrack, University of Colorado Health Sciences Center, Denver, USA. Professor Shosaku Numa, Departments of Industrial Chemistry and Molecular Genetics, Kyoto University, Japan. Dr Minoru Oda, president, Institute of Physical and Chemical Research, Japan. Professor emeritus Toshitsugu Oda, director, National Medical Center, Japan. Dr T S Okada, director-general, National Institute for Basic Biology, Okazaki, Japan. Professor Martin Rees, Institute of Astronomy, University of Cambridge, UK. Professor Frank H T Rhodes, president, Cornell University, Ithaca, New York, USA. Sir Mark Richmond, vice-chancellor, University of Manchester, UK. Professor Jonas Salk, Salk Institute, La Jolla, California, USA. Dr Maxine Singer, president, Carnegie Institution, Washington, DC, USA. Sir Peter Swinnerton-Dyer, chairman, University Grants Committee, London, UK. Professor Howard Temin, Department of Oncology, University of Wisconsin, Madison, USA. Professor Susumu Tonegawa, Center for Cancer Biology, Massachusetts Institute of Technology, Cambridge, USA. Lord Todd, past president of Royal Society, master of Christ's College, Cambridge, UK. Dr Alexander Varshavsky, Department of Biology, Massachusetts Institute of Technology, Cambridge, USA. Professor A Wada, Department of Physics, Tokyo University, Japan. Professor emeritus Itaru Watanabe, Keio University, Japan. □

Giant eye on the sky to reopen

Washington

THE Carnegie Institution of Washington, which has operated an astronomical observatory on Mount Wilson, outside Los Angeles, since 1904, last week handed over the site to the Mount Wilson Institute. This private non-profit organization will continue the current programme of solar observations, and also intends to restore to working order the 100-inch Hooker telescope, out of use since 1985.

The Carnegie Institution decided five years ago to spend more money on Las Campanas, Chile, a high-altitude, dark-sky site especially suited for cosmological studies of distant galaxies, by closing down the 100-inch telescope at Mount Wilson, whose capabilities had been limited by light pollution and smog in the Los Angeles basin. But Mount Wilson is an excellent

site for bright-star astronomy. Arthur Vaughan, head of the Institute, says the refurbished and reinstrumented telescope will be used for long-term projects on Sun-like activity and variability in nearby stars.

Vaughan expects it will cost about \$750,000 a year to operate the observatory. Initially, much of this money is expected to come from the National Science Foundation and other agencies. But Vaughan hopes to give the new observatory some independence by seeking funds from private foundations, local government and industries. He intends the Institute to work in educational programmes, and to be available to the local community. Before the advent of the National Science Foundation in the 1950s, astronomers were adept in attracting private grants and gifts and, says Vaughan, they must "relearn that talent". David Lindley

Complacent reviewing

SIR—In the course of preparing a review article I have been struck by the number of incorrect citations of the literature and of complacent reviewing in both review journals and regularly reviewed research articles.

First, in a recent review in *Bio Essays*¹, we can read that the human ferritin H gene has 4 exons and the human ferritin L gene has 3. The reference quoted is correct — but the article in question clearly states that there are 4 exons in the gene of human L ferritins, covering the same positions of the amino-acid sequence as they do in all mammalian ferritin genes so far sequenced. In a recent review in *Physiological Reviews*², the reference to a paper on intestinal ferritins (of which one of the authors of the review is the first author) lacks one of the co-authors. In *Annual Review of Biochemistry*³, we find a table which gives the same literature reference for the sequences of horse and human spleen apoferritins (they were published in two distinct articles) and the affirmation that pig liver apoferritins present three distinct sequences, when spleen was the organ of origin.

The same malaise can be found in original reviewed articles (I leave aside the innumerable errors in reference citations). It sometimes seems that the reviewer has accepted the article for publication without reading the text. This must be the explanation for the fact that in a recent *Journal of Biological Chemistry* article⁵ on plant ferritins, there is a figure which presents the determination of the molecular weight of the plant ferritin on the basis of one molecular weight marker, horse spleen apoferritin, and which arrives, without comment, at a molecular weight for the oligomer of 540,000, which corresponds to an oligomer composed of 19 subunits whereas all of the well characterized ferritin structures have a 24-subunit shell.

Both review journals and regularly reviewed scientific publications must ensure that both the references cited and the scientific content of their published manuscripts are coherent and respect the integrity of the scientific literature. It would perhaps be best that review journals use peer review to ensure that the author does not inadvertently perpetrate errors which will be autopropagated by the authors of original scientific texts who, in general, rely on reviews to furnish the literature citations that they employ. And reviewers of original articles should avoid recommending acceptance of an article they have not had time to read properly.

As responsible researchers, we should not allow complacent reviewing to take the place of the well-established methods which need both time and patience, both

to write good review articles and to review original articles. What we must do, as Sydney Brenner advised his student, is not to Xerox the literature, but to *neurox* it — “hold the page in front of your eyes and you let it go through there into the brain”⁶.

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Einstein in Zurich

SIR—The ‘Science in Switzerland’ supplement mentions that the first professors at the ETH were “refugees who fled prison sentences or political repression in Germany after the failed 1848 rebellion” (*Nature* **336**; 332, 1988). The writer failed to mention another young German refugee who applied for admission to the ETH, Zurich, in 1895, when two years below the required age. This refugee, Albert Einstein, failed the entrance examination then. But in the summer of 1896, he enrolled in the natural science degree programme of ETH, Zurich, and passed the final examination in August 1901. After that, he was passed over at the ETH for a university assistantship.

SACHI. SRI KANTHA

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Still active

SIR—Your statement that the Sacramento Peak Solar Tower has been closed (*Nature* **336**, 340; 1988) is unfortunate, and fortunately incorrect. In fact, in spite of a catastrophic decrease in the National Science Foundation's support for solar physics, as well as other disciplines, the National Solar Observatory has successfully struggled to maintain a vigorous programme of research, as well as support of visiting astronomers, at the Sacramento Peak Tower.

The tower, constructed in 1968 by the United States Air Force, and its associated instrumentation continue to provide a productive facility for studying the small-scale, inhomogeneous structure of the Sun. Work at Sacramento Peak, and elsewhere, during those 20 years has shown

that this structure is responsible for the varying solar magnetic activity as well as the heating of the outer solar atmosphere to millions of degrees. This rich variety of challenging theoretical and observational questions continues to make solar physics an exciting field in its own right, in addition to its role as the source of important solar-terrestrial interactions and as the Rosetta Stone for stellar astronomy.

This is not to undercut your main point that, because of the low level of support, existing solar physics facilities are not used as effectively as they should be, and that major investments in productive scientific facilities are continuously being threatened with closure. Fortunately, particularly with an impressive maximum of solar activity approaching, pressures to close Sacramento Peak have not succeeded. If the international hue and cry that these attempts have prompted in the past is any measure of the demand for the facility, the Sacramento Peak Tower should be the focus for solar physics research for many years to come.

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SDI defended

SIR—I would like to correct some factual errors in the leading article on the Strategic Defense Initiative (*Nature* **335**, 575; 1988). First, the SDI director is Lieutenant General James Abrahamson; he is not a lieutenant-colonel. Second, President Reagan delivered his SDI speech on 23 March 1983, not 13 March 1983.

SDI is a long-term research and development programme attempting, at least in part, to separate fact from fable and fiction so that a future administration will be able to make an informed judgement whether to proceed with a Strategic Defense System (SDS). In order to accomplish this most important endeavour, we need the support of peace- and freedom-loving people everywhere. Indeed, our Western civilization, on both sides of the Atlantic, could be saved thanks to a concept orders of magnitude superior to the present one based on MAD (Mutually Assured Destruction). If SDI is destined to fail, then so be it. At present, though, we should give it our best shot before giving up even, possibly, our lives. I hope you concur with my rather meagre attempt at scientific objectivity.

ROY D. NORTH

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only. □

Ambitions for Lake Baikal

A scheme for creating an international research centre seems to break new ground in the pattern of Soviet collaboration with the West, and deserves warm encouragement.

ONE of Mikhail Grachev's dreams seems well on the way to becoming true. Grachev is the energetic new director (since 1986) of the Institute of Limnology of the Siberian Branch of the Soviet Academy of Sciences near Irkutsk, the south-east Siberian city nearest to Lake Baikal. The institute is now moving even closer to the world's largest body of fresh water. While Grachev has been given generous support since the outset of his tenure, he has always known that a complement of 120 scientists would be insufficient for the work that needs to be done.

Just over a year ago (see *Nature* 329, 802; 1987), he was dreaming of attracting people from outside the Soviet Union to exploit the opportunities of Baikal. Now, in an astonishingly short time by Soviet standards, he is launching an international research centre at Listvyanka, on the edge of the lake. In its constitution, the centre will be unique by Soviet and, perhaps, by any standards.

The crux of the idea is that overseas research organizations, either academies or grant-making agencies, should become partners with the Institute of Limnology in the financing and direction of the centre, acquiring an equal voice on scientific policy. While the long-term objectives would be agreed at the outset, each year's research programme would be determined by the international council on the basis of specific proposals and the assessments of international panels of referees. It is hoped that overseas members will contribute up to a third of the cost of the centre (roughly 50 million rubles), ambitiously intended to include kindergartens for visitors' children as well as social facilities for making Siberian life tolerable for long-stay visitors. Grachev says there will be no charges for running costs, which will be borne ultimately by the Academy of Sciences, but project participants will have to get themselves and their families as far as Moscow. The proposed charter even includes provisions for the return of contributions by the founder-members if, for some reason, the centre is wound up.

Grachev, who bubbles with enthusiasm, is anxious that the operational management (which, for legal and practical reasons, must be Soviet) should be seen to be responsible to the international council: he plans that there should be an executive officer (appointed by the council) responsible for carrying out policy decisions. Otherwise, principal investi-

gators will direct their own research programmes in the usual way. While the Institute of Limnology will continue its independent existence, much of what it does will consist of support for the international centre.

The outline plan has already been approved by the praesidium (executive council) of the Soviet academy. Dr V. A. Kapyug, the president of the Siberian Branch of the academy, a crucial backer, has been commending the plan on recent visits to Western Europe and the United States. Grachev's model is the Woods Hole Marine Biology Laboratory, a familiar magnet for long-stay visitors, but the planned constitution probably resembles most closely that of the International Centre for Insect Physiology and Ecology at Nairobi. Grachev is hoping that there will soon be some indication of overseas commitments.

Will this optimism be justified? Even as a once-and-for-all capital contribution, a third of 50 million rubles is a considerable sum, especially when limnologists in the West are being starved of funds. Much will turn on Grachev's case that Lake Baikal, acknowledged to be a distinctive natural phenomenon, may be made to yield general understanding.

Here is a version of Grachev's case, culled from a breathless summary over dinner a few days ago. First, Baikal is unique not only by its size (literally a fifth of the world's fresh water) but it is also (partly for that reason) the most pure. The margins of the lake remain mostly in their primitive condition. Moreover, the water regime is distinctive. There is one major inlet and one outlet, both in the southern third of the 1,000-km inland sea. Were it not for the winter covering of ice, the vertical mixing that keeps the lake oxygenated throughout its great depth (up to 1,700 m) would not occur.

So there is a need for a mathematical model of the lake — a task Grachev reckons will take 20 years or more. The difficulty is not the construction and solution of mathematical equations but the measurements without which accurate modelling cannot be built. The nature of the lake, particularly its size and its substantially pristine condition, should yield a model that becomes a modeller's model.

On a similar tack, Baikal should be a valuable indicator of the spread of man-made pollutants over great distances.

With the exception of the two now-notorious cellulose plants due to be closed in 1993, there has never been any substantial industrial activity within the watershed, so that heavy metals and artificial chemicals found in the lake must have made their way there by other means.

The great seal infection of 1987 is a puzzle much of this kind. The Baikal seals were stricken a season earlier than those of the Baltic and the North Sea. The viruses responsible appear to be identical. By what means did the virus spread across Eurasia? Most probably, birds. Where did it come from? Nobody knows. And what is the fate of the Baikal seals? Recent investigations show that roughly 70 per cent of them carry antibodies to the virus, suggesting that mortality in 1989 will be largely restricted to pups in the post-weaning stage.

Lake Baikal's freshwater seals are a distinct species whose provenance is unknown, but that is true of more than a third of the 1,700 species of plants and animals recorded in the lake. Grachev's own interests (he is a chemist turned nucleic acid specialist) prompts a third line of investigation — the use of DNA-sequencing techniques to reconstruct the genetic differentiation of life-forms in the lake during the past 25 million years or so. There are a score of molecular biologists already, but they can hardly scratch the surface of a gigantic problem.

Meanwhile, Baikal's sediments, almost literally untouched, should provide splendid opportunities for palaeo-specialists. But there is apparently still dispute about the typical thickness of the deep sediments — 500 m or three times as much? An expedition with the University of Hamburg plans a start on this work during the coming ice-free season.

Curiously, even the tectonic structure of the region is inadequately known by modern standards. Although the geology has been mapped for half a century, much of what is known of the rift is inferred by analogy from elsewhere, principally East Africa. That the rift is active is plain from the seismic activity of the region. Will the new centre give geophysicists the chance to instrument the rift as carefully as, say, the San Andreas fault in California?

In Grachev's book, anything is possible. To have hatched out the idea of an international centre at Lake Baikal in such a short time suggests that what is possible is likely also to happen. **John Maddox**

AIDS

HIV and Kaposi's sarcoma in mice

Robin A. Weiss

ONLY three months ago, I reported¹ in News and Views on the promise of novel systems for studying aspects of the pathogenesis of the human immunodeficiency virus (HIV) in mice and rabbits. Such is the pace of AIDS research that two new reports have already appeared in the 23 December issue of *Science* to substantiate that promise. I.L. Weissman's and J.M. McCune's group at Stanford has now demonstrated² that immunodeficient mice reconstituted with human lymphoid cells can be infected with HIV-2, and M.A. Martin's group at NIH has reported³ on the fate of transgenic mice inheriting a complete genome of HIV.

Only two species of animal, chimpanzee and man, are readily susceptible to HIV infection, and only man so far produces AIDS symptoms when infected. But mice with severe combined immune deficiency (SCID) will accept engraftment, proliferation and differentiation of human haematopoietic cells. This was recently reported for lymphoid cells^{4,5} and has now been extended⁶ to myelo-monocytic stem cells in mice recessively homozygous for three distinct immunodeficiency genes, *b2m*, *gld* and *lck*. In their new work², Namikawa *et al.* inoculated HIV directly into human lymphoid xenografts in SCID mice and demonstrate HIV replication. The number of cells expressing HIV by *in situ* hybridization increased progressively up to 8 weeks post-infection, which is when the article went to press. More cells expressed viral RNA than viral antigens, although HIV proteins could be detected, as well as characteristic multinucleated, syncytial cells, especially in the medulla of the implanted human lymph node or thymus fragment. Thus, the authors demonstrate *in vivo* HIV infection of mice carrying human lymphoid tissues. To my mind, the experiments so far described represent a kind of *in vivo* 'organ culture' for HIV infection, and it remains to be seen whether the system will be exploitable for studies of pathogenesis or protection against infection.

Leonard *et al.* have generated³ transgenic mice incorporating entire, infectious genomic clones of HIV. This has been a controversial project and has required extraordinary levels of containment, lest a mouse should escape and perhaps pass on the HIV genome by breeding with wild mice. Unfortunately, a recent accident with the ventilation system in the containment facility has led to the loss of all the transgenic mice (see *Nature* 336, 613; 15 December 1988).

The authors show that the HIV genome

does become incorporated into the tissues and germ-line of the mice inoculated as pre-implantation embryos with HIV DNA. Curiously, none of the founder mice with proviruses expresses HIV, but one female mouse mated with a nontransgenic animal produced several offspring whose tissues contained infectious HIV particles. These mice developed a runting syndrome, expressed rescuable HIV and died before reaching maturity. The runting was accompanied by severe epidermal hyperplasia, particularly of the non-furry extremities (tail, paws, ears and snout), and by a grossly enlarged spleen, lymph nodes, lymphoid infiltrates of the lung and thymic involution. There was no evidence of opportunistic infections, which is not surprising as the mice were maintained in a germ-free environment. Neither was there gross evidence of neurological dysfunction, nor of HIV expression in the brain. No depletion of CD4⁺ T lymphocytes was noted, but in the affected lymph nodes there was an increase in CD8⁺ T cells.

Leonard *et al.* argue that a low level of HIV expression in cells such as macrophages (one of the cell types infected in human AIDS) might be sufficient to elicit the production of cellular factors that contribute to the runting syndrome. This argument may explain how lentiviruses like HIV in humans and visna-maedi virus in sheep can be severely pathogenic with low levels of virus present in the body. It will certainly be interesting to measure the production of proteins such as tumour necrosis factor (made in the macrophages)

in these mice. The fact that the skin and lymphoid tissues showing abnormal growth are largely those expressing the HIV genome suggests that, in the main, such indirect factors act locally.

My own view is that transgenic mice expressing HIV genes singly or in combination, rather than the whole genome, will be more informative in probing the complex pathogenesis beyond the immediate destruction of infected cells⁷. Vogel *et al.*⁸ have already reported on mice transgenic with the *tat* gene of HIV. These mice also display hyperplastic skin lesions, though these were described as endothelial and spindle cell proliferation, more reminiscent of Kaposi's sarcoma (KS) than the epidermal thickening in the transgenes of Leonard *et al.*³. Interestingly, the KS-like lesions in *tat* transgenes occurred predominantly in male mice, just as with KS in humans, whereas the entire HIV genome in transgenes affects both males and females.

Kaposi's sarcoma also features in two papers from R.C. Gallo's laboratory^{9,10} that have been heralded for some time by his pronouncements at conferences. Nakamura *et al.*⁹ found that certain T-cell lines transformed by human T-cell leukaemia viruses release paracrine growth factors that stimulate the proliferation of KS-derived cells. Interestingly, the KS cells also respond to tumour necrosis factor, so one of the factors that may exacerbate wasting disease may also contribute to KS. Salahuddin *et al.*¹⁰ show that the stimulated KS cells then release their own factors, including strongly angiogenic activity that engenders KS-like proliferation of murine cells upon implantation in nude or beige mice. These findings lend weight to the proposition put forward five years ago by Costa and Rabson¹¹ that KS in AIDS is not a true malignancy of clonal origin that has become disseminated throughout the

100 years ago
NOTES

A MOVEMENT has been started in Norway for the despatch of an Expedition to the North Pole, and it is proposed that the leadership shall be offered to Dr Nansen. Those who are arranging the plans maintain that no other country could furnish such a crew of experienced and hardy ice men as Norway, and that a winter or two in the Arctic regions would affect these men very little. The intention is to reach the Pole by way of Franz Josef's Land, a route advocated by the most experienced Norwegian Arctic travellers. *Ski*, which have played such a prominent part in the Nordenskiöld and Nansen Greenland expeditions, would no doubt again be of great service.

IN THE January number of the *Kew Bulletin* there is a most interesting paper on the coca-plant, to which considerable attention has lately been devoted, mainly because of the valuable properties ascribed to one of its alkaloids, called cocaine, as a local anaesthetic. It appears that since the discovery of the anaesthetic properties of cocaine the demand for coca-leaves in South America has considerably increased for export purposes. A distinct loss in the alkaloids generally has been noticed during the transit of the leaves to this country; and latterly it has become the practice to extract the alkaloids from the leaves in South America, and export to the United States and Europe a crude preparation. The demand for coca-leaves has, therefore, fallen off, and the cultivation of the coca-plant in our tropical colonies will probably never assume large proportions. South America is able to produce such enormous quantities of coca-leaves that the one-eightieth part would be sufficient to swamp the cocaine markets of the whole world.

From *Nature* 39, 256; 10 January 1889.

body; rather it may represent a local hyperproliferative response to unknown angiogenic signals.

A report by Lo and Liotta¹² claiming that transfection of KS 'oncogene' into NIH-3T3 cells leads to the formation of murine KS-like neoplasms may also represent a growth-factor gene. Further characterization of this interesting phenomenon of DNA transfection leading to murine tumours of the same histopathological type as the human KS has not been forthcoming. Della Bovi and Basilico more thoroughly analysed¹³ a transforming gene for NIH-3T3 cells derived from human KS and found that it is derived from sequences adjacent to the *c-fms* oncogene. The authors point out, however, that this gene may have been 'activated' during the preparation of the DNA, as there is no evidence of sequence rearrangement in the original KS DNA. Of course, if the KS cells are not clonal, it would be difficult to detect.

The epidemiology of KS remains something of an enigma. In AIDS it is seen in association with sexually transmitted HIV, less frequently among drug abusers,

and is virtually unknown in haemophiliac AIDS. The incidence of KS is falling among homosexuals with AIDS. Although the primary aetiological agent for KS awaits discovery, the manipulation of KS cells in culture and xenograft with the growth factors developed by Gallo's group should help to delineate further properties of this fascinating tumour. □

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Solar System

Planetary planarity

David W. Hughes

THE Solar System is flat. But is this a relic of the origination process, the planets being formed out of a flat disk of accreting planetesimals orbiting in the equatorial plane of the cloud of gas and dust that surrounded the newborn Sun? Or did the system become flat later, this flatness being imposed by the gravitational dominance of Jupiter, the most massive planet? Also, does the flatness of the Solar System limit the size and the distance of nearest approach of the star which has passed closest to the Sun in the lifetime of the Solar System (4,570 million years)? These questions are investigated by Donald E. Morris and Thomas G. O'Neill in a recent issue of the *Astronomical Journal* (**96**, 1127–1135; 1988).

All planetary orbital parameters vary with time owing to their mutual gravitational interactions. By using a computer to trace the Solar System back 100 million years it has been found, for example, that the inclination of the orbit of Jupiter to the orbit of the Earth varies in a quasiperiodic fashion between 0.17° and 0.57°. In the case of Neptune, the most weakly bound of the planets, the range is 0.46°–0.86°. The variation in the orbital eccentricity of these planets is 0.025–0.062 and 0.0001–0.023, respectively. No secular trends have been found. These findings support the relic theory and indicate that Jupiter has done no more than influence

the size of the range of variability.

The small mean inclination 0.69° and mean eccentricity 0.01 of Neptune could be accounted for in two ways. They could be relics of the formation process, but in addition we would have to conclude that they have not been affected by a star passing close to the Solar System since that formation. Or they might have initially been much larger and have subsequently been reduced by a passing star. Morris and O'Neill show that the latter of these two possibilities is most unlikely and indicate that passing stars cannot have changed the inclination and eccentricity of the planets by more than about the mean values that they have today. So the stellar-induced changes in the orbital velocity of Neptune and Uranus can have been no more than 0.084 and 0.3 km s⁻¹, respectively, during their lifetime.

The change in planetary velocity introduced by a passing star can be easily calculated and is a function of the star's velocity, mass, miss-distance and the encounter geometry. The change in the velocity occurs in a time that is much shorter than the planet's orbital period. Also a star that passes on a path parallel to the planet–Sun direction produces a much smaller perturbation than one that passes perpendicular to that line. By using the known stellar spatial density and velocity distribution in the solar vicinity, Morris

and O'Neill calculate how close stars have to come to the Sun in the past and find that the inclinations and eccentricities of Saturn and Uranus, and the eccentricity of Jupiter, almost certainly have not changed significantly because of stellar encounters since the formation of the Solar System. Even for Neptune the probability of a stellar encounter capable of changing its inclination and eccentricity by more than half its present value is less than 3 per cent.

These limits indicate that no star with a mass greater than 0.1 solar masses has passed through the Solar System during the system's lifetime and no object with a mass more than three times that of Jupiter has passed within the orbit of Earth. Also no weakly bound object (such as a black dwarf star or a giant comet in a distant but eccentric orbit around the Sun) of mass greater than 0.01 solar masses has passed through the system. This, however, does not rule out the existence of a Nemesis-like solar companion as long as it always stays at great distances from the Sun. Remember also that the Sun was initially a member of an open cluster of stars. The Pleiades and Hyades are typical examples of such clusters. Some 10⁸ years ago the Sun escaped from its parent cluster, and in its present position in the galactic disk the special density of stars (3.3 × 10⁻⁴² stars km⁻³) is about one third its initial value. In the cluster, close stellar encounters would be three times more common.

There is one more significant conclusion. Passing stars cannot be invoked to strip away outlying planets, especially the as yet unobserved Planet X that is a favourite of astronomers trying to explain apparent minor perturbations in the orbits of the outer planets. So the boundaries that the Solar System has today are the boundaries that it had initially. No big planets beyond Neptune have been lost.

Needless to say, I have been discussing stars getting *really* close to the planets. Significant numbers will have passed through the Oort cloud of comets, a cloud that occupies a spherical shell of inner radius 10,000 astronomical units (AU; 1 AU equals the mean radius of Earth's orbit) and outer radius 100,000 AU centred on the Sun (see for example Stern, S.A. & Shull, J.M. *Nature* **332**, 407–411, 1988).

I still, however, have one niggling worry. If the planetary planarity is primordial and the planets and the Sun were formed at the same time and from the same cloud of gas and dust, why does the planetary plane not coincide with the equatorial plane of the Sun, instead of being inclined to it by 7.25°? Maybe 7.25° is close enough to 0.0° to be negligible. But maybe not. □

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Parasitology

Seventy-five years of solicitude

Anne Keymer and Don Bundy

IN 1913, the Rockefeller Foundation accepted the "urgent invitation" of the British Colonial Office to undertake a worldwide campaign of hookworm control. Six years later, the foundation's researchers had obtained estimates suggesting that almost 1,000 million people, more than half the world's population at that time, were infected. They concluded, with a hint of ambitious philanthropy, that "this one disease may go far to explain the retardation of backward people" (Ettling, J. *The Germ of Laziness*, Harvard University Press, 1981). The campaign achieved notable successes (Fig. 1) and laid the foundation of modern helminth epidemiology, but this momentum was not sustained beyond its lifetime. Global estimates of hookworm infection still stand at 1,000 million cases, though these now represent only one quarter of the total world population. The results of research reported at a recent meeting* suggest that the time has come for renewed effort.

Hookworm disease is the term traditionally used to describe infection with either of two species of intestinal nematode, *Necator americanus* or *Ancylostoma duodenale* (Fig. 2). But there are important differences in the reproductive strategies of the two species (G. A. Schad, University of Pennsylvania): *A. duodenale* is more pathogenic than *N. americanus* (as judged by the rate of associated blood loss) and has a higher reproductive rate. Human volunteer studies suggest, as might be predicted, that *N. americanus* has the longer lifespan (E. A. Ottesen, National Institutes of Health). *A. duodenale*, unlike *N. americanus*, can infect the host orally as well as by skin penetration, and is capable of arrested development in the tissues of the host and perhaps of trans-mammary transmission from mother to offspring. These and other differences between the two species almost certainly result in divergent epidemiological patterns, the description of which awaits species-specific diagnostic tests for the eggs liberated in the faeces of infected patients, and for larvae in the contaminated environment. The possibility that community control may shift the balance between the two parasites, for example by selecting for arrested larvae resistant to the effects of anthelmintic drugs, has not yet been adequately considered.

The best prospects for a species-specific diagnostic test seem to be the heat-shock proteins that constitute species-specific

immunodominant antigens in several human disease agents (N. Agabian, University of California, San Francisco). Although there has been some progress, studies of the functional significance of immune responses against human hookworms have been hampered by the absence of epidemiologically defined sera. The surface antigens of *N. americanus* are highly immunogenic, and may shield the underlying collagen which constitutes the main structural component of the parasite's cuticle. Analysis of the sera already available indicates a marked diversity in antigen recognition between patients from endemic areas. If the antibody responses against cuticular

IMAGE
UNAVAILABLE
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REASONS

Fig. 1 A 7-year-old Tamil boy before treatment for hookworm disease (left). His weight was 32 pounds. Right, the same boy 6 months later, after being cured. Photograph taken in 1920 by W. Rose.

collagen observed in some patients indicate immunologically mediated parasite damage, the prospects for vaccine development may be better than has previously been supposed (D. I. Pritchard, University of Nottingham).

Hookworms produce secretory antigens which may be of functional significance. Acetylcholinesterase, for example, may modulate the effects of the host's immune responses as well as acting as a biochemical holdfast for the parasites. Proteases which cleave IgA (one of the putative immunological effectors in the intestine) have also been demonstrated, and infected patients may have reduced levels of intestinal IgA which return to normal after treatment to remove intestinal worms (Ottesen). Determining the role of these responses in naturally acquired infection depends on further understanding of immunology and epidemiology.

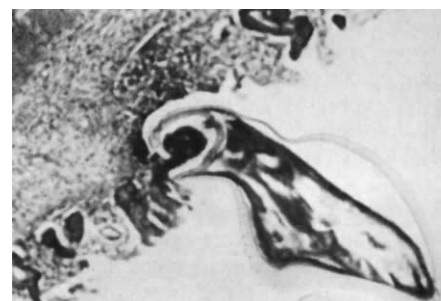


Fig. 2 Section through the lumen showing part of the host intestine in the mouth of *A. duodenale*. The patient died of intestinal blood loss.

Hookworms are known to exert various insidious effects on health, and have been widely associated with the iron-deficiency anaemia which affects 350 million women of child-bearing age. The role of hookworm infection as a contributory factor in the maternal and fetal morbidity associated with anaemia is now beyond dispute (D. W. T. Crompton, University of Glasgow). There is clearly a correlation between hookworm infection and impairments in growth and physical fitness. A study of Kenyan schoolchildren (L. S. Stephenson, Cornell University), for example, demonstrated a significant difference in growth rate between children allocated at random between placebo and anthelmintic treatments. The improvement in weight gain in the treated children (about 1.3 kg) is correlated with pretreatment hookworm egg counts, occurred despite continual exposure to reinfection and incomplete cure, and demonstrates one of the benefits that might accrue from effective control.

Mortality from acute infectious diseases is gradually coming under control, which means that morbidity from chronic infections such as hookworm assumes greater importance. Control of this morbidity is emerging as a new challenge to public-health workers in developing countries. The revolution in primary health care and the development of safe and effective broad-spectrum drugs provide new opportunities to meet this challenge in a cost-effective way, notably by the integration of the control of hookworm and other chronic nematode infections in existing health-care delivery systems. National schistosomiasis control programmes, for example, provide an existing infrastructure for disease surveillance and drug delivery, and often a sanitation component which may influence the transmission of a wide range of enteric infections. Whatever the combination of interventions — chemotherapy, sanitation or even vaccination — current research in epidemiology greatly strengthens the prospects for an integrated strategy. □

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* Hookworm Disease: Current Status and New Directions, organized by G.A. Schad and K.S. Warren. Rockefeller Foundation, Bellagio, Italy, 26–30 September 1988.

Nuclear fusion

Insulating hot plasmas

R.S. Pease

EXTENSIVE progress has been made at four large nuclear-fusion experiments (the Joint European Torus, JET, at Culham in the United Kingdom, TFTR at Princeton University, D-III-D at San Diego and JT-60 in Japan) according to the results reported at a recent meeting*. With plasma temperatures of up to 350 million kelvin (K) and fusion output powers up to 60 kilowatts, these assemblies, now half way through their experimental programmes, are achieving the plasmas with temperatures and densities forecast at the design stage. They are meeting their objective of elucidating the physics and engineering of the magnetic confinement of high-temperature plasmas in conditions close to those envisaged for commercial controlled-fusion reactors. But the experiments have not achieved simultaneously the thermal insulation and high temperature needed for net energy output from fusion reactions in the optimum fuel, a 50-per-cent mixture of the hydrogen isotopes, deuterium and tritium. This could come towards the end of the experimental programmes, if all goes well.

The prospect of generating energy by fusing together the nuclei of hydrogen isotopes and other light elements has stimulated physicists and engineers to devise several schemes for containing controllably the hot ionized gases involved. Each of the experimental assemblies mentioned above is a tokamak, that is a toroidal electrical discharge stabilized by a strong magnetic field (Fig. 1). Discharge currents exceeding about 1 million amps (MA) are needed to achieve the required conditions; the largest currents are obtained in JET, where up to 7 MA flows through a deuterium plasma (density $\approx 3 \times 10^{19} \text{ m}^{-3}$; R.J. Bickerton, Culham). In all the installations, additional heating is provided by radio-frequency heating and by the injection of energetic neutral atoms at powers up to 30 MW.

The most critical issue being addressed is the thermal insulation of the high-temperature plasma provided by the magnetic field embedded in it. The gross energy confinement time τ_E defined as the quotient of the thermal energy of the plasma and the power input required to sustain it, is a measure of this thermal

insulation, and has to be of the order of 1 s in a power-producing magnetic fusion reactor. In these large experiments, τ_E is in the range 0.1–1 s. The best values of 1.2 s were reported from JET at currents of 3–5 MA operated with additional heating and with an optimal magnetic field arrangement at the outer edge of the plasma (M. Keilhacker, Euratom).

These energy confinement times are promising only if they are accompanied by the appropriate values of the plasma

The energy confinement time τ_E is less than the ideal theoretical value arising from collisions between the ions and electrons in the plasma alone sometimes found in smaller tokamaks and at lower currents. The deficiency at the highest currents is about a factor of 100, and this large margin together with observed fluctuations in plasma density and magnetic field strongly indicates that an instability is present. There is no convincing consensus on the instability responsible, but it is almost certain that it depends on the details of the pressure, temperature and current-density profiles. It seems possible from the steep pressure gradients encountered locally in some conditions, that the global improvements mentioned above arise from regions where the local transport

coefficients are reduced towards the collisional values by the local suppression of the instability.

Many theoretical groups advanced hypotheses that drift-wave instabilities are the main source of trouble. Drift waves are the magnetohydrodynamic analogue in a magnetically confined plasma of surface-tension ripples on a fluid contained by gravity. They can be driven unstable by various causes, such as excessive temperature gradients. Calculations of the thermal losses that can arise from these instabilities give good agreement with experiment, *post-facto* and with fitted parameters. There is also experimental evidence of the

elimination of some of the losses when an ion-temperature-gradient driving term is reduced, in accordance with theory. It was also suggested that other driving terms, such as the presence of charged particles trapped in the field inhomogeneity, could be eliminated (T. Okhawa, San Diego). Others, (for example J.W. Connor, Culham) however, pointed to resistive instabilities due to the current flow in the plasma as a source of the plasma fluctuations and of the low values of τ_E .

The need to resolve the physics underlying the thermal insulation and to improve the values of τ_E was underlined by papers describing studies of the next tokamak experiments. These include the ITER (International Tokamak Experimental Reactor) studies by a quadripartite team (United States, Soviet Union, Europe and Japan), headed by K. Tomabechi (Japanese Atomic Energy Research Institute). If the confinement time scales as in Goldston's equation, then even with rather favourable assumptions about the coefficients in the equation, currents in these next experiments will have to be of the order of 20 MA to reach the desired self-sustained

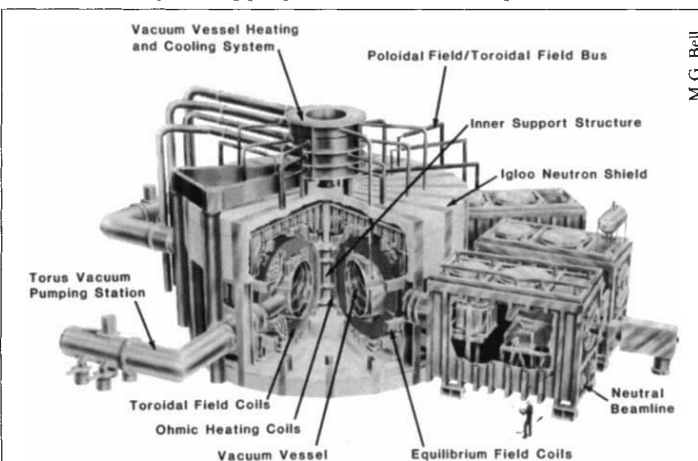


Fig. 1 Sketch of the Tokamak Fusion Test Reactor (TFTR) at Princeton University. The major radius R of the torus is 2.5 m, the minor radius, a , 0.85 m. JET is the largest current tokamak with $R=3$ m, and a D-shaped minor cross-section with radii 1.25–2 m.

number density and high temperature. At the required temperature of around 10^8 K, the ratio Q of the thermonuclear power output to the power required to sustain the plasma temperature is nearly proportional to the triple product of temperature T , number density n_0 and τ_E . The best value $n_0 T \tau_E$ reported from JET (Fig. 2) is $2.5 \times 10^{20} \text{ m}^{-3} \text{ s keV}$, for which the thermonuclear output is about one-tenth of the heating power. From TFTR, the maximum value obtained at 3×10^8 K is $3 \times 10^{20} \text{ m}^{-3} \text{ s keV}$. Both in JET and JT-60, the underlying variation of τ_E with various experimental parameters can be summarized by an empirical law suggested by R.J. Goldston (*Plasma Phys. cont. Fusion* **26**, 87–99; 1984): $\tau_E \propto I R^{1.75} a^{-0.4} H^{-0.5}$. That is, the thermal insulation improves with the current I and with the size of the machine (R is the major radius, a the minor radius), but decreases as more power H is used to heat the plasma. In all the machines, however, improvements of up to a factor of 3 could be obtained by various means discovered experimentally, mostly concerned with modification in detail of the profile of the density distribution or of the magnetic field at the edge of the plasma.

*12th international conference on plasma physics and controlled nuclear fusion research, Nice, 12–19 October 1988.

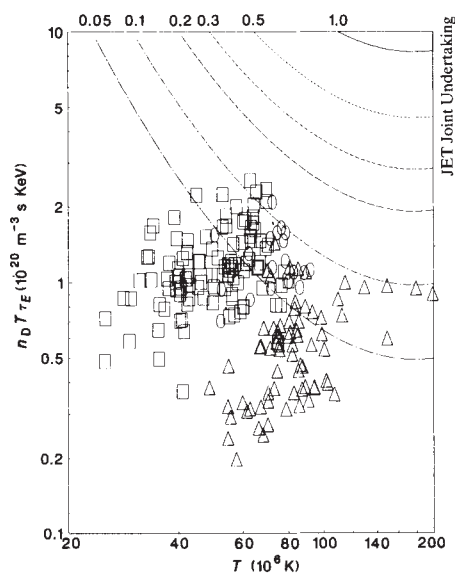


Fig. 2 Confinement observations on JET using deuterium plasma: the triple product $n_D T_E$ (see text) is shown as a function of central ion temperature T for a wide range of conditions. The 'H-mode' values ($\square$) are obtained with a magnetic separatrix at the edge of the plasma. $\triangle$, Low-density discharges; $\circ$, pellet-injection fuelling. Values of Q , the ratio of the calculated thermonuclear output of a 50-per-cent deuterium-tritium mixture to the total heating power needed to sustain the plasma conditions, are shown at the top (smooth curves).

fusion reaction (which needs $Q \geq 5$) in deuterium-tritium plasmas (R. Toschi, Euratom/P.H. Reubt, JET).

Encouraging results were reported on some parallel issues for fusion systems. In D-III-D, experiments have achieved good values of the plasma pressure without losing confinement in the sense of τ_E falling sharply (E.J. Strait). The average ratio of the plasma to the magnetic pressure reached is 6.8 per cent, at the favourable end of the range of theoretical prediction; for a commercial reactor one needs about 10 per cent to avoid excessive capital costs. From the same laboratory came a proposal (Okhawa) which, if substantiated by experiment, would enable the tokamak current to be sustained by a new and much more efficient current drive, using circularly polarized low-frequency electromagnetic waves travelling along the lines of force.

The use of superconducting electromagnets should also make sustained discharges possible. The large superconducting tokamak Tore Supra at Cadarache in France, which has carried currents of 500 kA in its initial runs, is designed to sustain a current of 1.7 MA for 30 s. But the present record is held by the Triam superconducting tokamak at Kyushu University, in which a 23-kA discharge was sustained for 3 min (S. Itoh).

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Sexual selection

Is bigger always better?

Mark Kirkpatrick

THERE IS NO such thing as a free lunch. At least that is the assumption under which evolutionary biologists, together with engineers, theologians and Reaganites, often labour. Thus the report from von Schantz *et al.* on page 166 of this issue¹ that male pheasants with larger spurs both attract more females and have high survival is surprising — it appears that these males are getting mating advantage without paying a price in viability. The situation is made all the more remarkable by the finding that females mated to large-spurred males have more offspring. These results are important on two levels: they provide a mystery about the evolution of a male secondary sexual trait (the spurs); and they fuel a controversy over how preferences for male traits evolve in the first place.

First, the mystery. Darwin^{2,3} built his theory of sexual selection around a notion of tradeoffs. He argued that traits such as the spurs of male pheasants promote mating success by attracting females and excluding male rivals but are disadvantageous to survival. After all, if these traits improved survival the females would have them too. Von Schantz *et al.* test this argument in a natural population by observing the ability of male pheasants to attract females to their territories. Using both natural variation and an elegant experimental manipulation, they show that males with larger spurs are more attractive to females. (Surprisingly, spurs do not appear to affect dominance in male-male competition.)

Large spurs in addition are correlated with increased male survival, although here it is less clear that spur length *per se* rather than some correlated variable is responsible. Assuming, as the authors do, that longer spurs themselves do increase male survival, it appears that survival and reproduction unanimously favour larger spurs. This is unusual: many or most male traits that increase male attractiveness do appear to decrease survival as assumed by Darwin⁴. The mystery, then, is why male spurs are not very rapidly increasing in size over evolutionary time, and why females lack them entirely. The two obvious possibilities are that selection is inconsistent and favours small spurs in other years, or that there is no additive genetic variation for spur length in the population that will allow the character to evolve. These obvious possibilities, however, have obvious shortcomings: there is no *a priori* reason to think that these data on selection are atypical, and there is no precedent in which a male secondary

sexual character has been found to lack genetic variation.

Second, the controversy. In framing his theory of sexual selection, Darwin traded one difficulty for another. He postulated that certain male traits evolve because females prefer to mate with males that carry them, but failed to explain why the female mating preferences evolve in the first place. There are two schools of thought on this question^{4,5}. The 'good-genes' school maintains that evolution will generally produce female mating preferences for traits that increase male survival.



A male chinquis peacock pheasant *Polyplectron bicalcaratum* showing off its double set of spurs in Darwin's *Descent of Man*³.

Genes for such preferences are expected to spread as the trait they prefer does because of a genetic correlation that spontaneously develops between the preference and male trait. The 'nonadaptive' school, on the other hand, argues that other evolutionary forces acting on the preferences will overwhelm the good-genes factor and often establish female mating preferences for traits that decrease male survival. Various forces are capable of doing this, for example selection on females to avoid mating with the wrong species, or selection on female sensory systems in other contexts that establishes mating 'preferences' as a side-effect.

The pheasant data provide an example clearly consistent with the good-genes view: females prefer males with larger spurs, and larger spurs are correlated with high survival. Furthermore, females mated to large-spurred males successfully hatch more chicks. This intriguing observation is consistent with a critical assumption of the good-genes hypothesis: that

there be heritability for the male trait which improves survival and attracts females. This finding is reminiscent of Partridge's⁶, showing that male *Drosophila* that succeed in mating competition also tend to produce larvae with high survivorship, and of Watt *et al.*⁷, showing that certain genotypes in male *Colias* butterflies receive both a mating and a survival advantage.

Supporters of the nonadaptive view, however, will be quick to point out that showing that mate choice in pheasants is consistent with the good-genes view falls short of showing that the good-genes mechanism is actually at work. First, the observation that females mated to long-spurred males fledge more offspring has not been tied to a genetic effect from the fathers. It is possible, for example, that these females are laying more eggs or are giving them better care as a consequence of mating with these males. This possibility is suggested by Burley's⁸ work on zebra finches showing that mating with a preferred male causes females to expend more parental care than they would otherwise.

Second, and much more difficult, is the point that these data focus on the male trait, while the controversy revolves around the evolution of the female preference. Whereas the pheasant data are consistent with the good-genes hypothesis, they are also consistent with alternatives. A definitive resolution of the controversy may have to wait for a system in which the evolutionary forces and inheritance of female preferences themselves can be studied. Until that goal is realized, we may be able to show how some males get a free lunch, but not why females choose to sit at their table. □

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Zeolites

Multidimensional large pores

Mark E. Davis

ZEOLITES and zeolite-like materials comprise a broad range of microporous, crystalline solids whose main uses are as adsorbents, catalysts and ion exchangers. These solids have tunable properties such as pore size, shape and capacity, and lead one to believe that designer catalysts are possible through the molecular engineering of zeolite structures (frameworks). Brunner and Meier on page 146 of this issue¹ report findings which provide insights for future synthesis directions which could lead to new zeolites with properties that are not currently available.

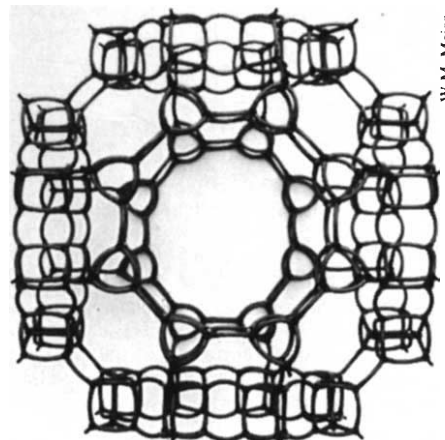
The basic building blocks of zeolites are tetrahedra of SiO₄ or AlO₄, which connect to form complex networks of one-, two- or three-dimensional molecular-sized tunnels. The pores can be used to sort molecules, and their surfaces to catalyse reactions.

Brunner and Meier show that there is a correlation between the minimum framework density (FD) defined as the number of tetrahedral atoms (T-atoms) per cubic nanometre and the smallest ring size in the framework. Clearly, the FD is related to the void volume of the crystal: as the FD decreases, the void volume or capacity for adsorption increases. The minimum known FD is 12.5 which corresponds to the void occupying just over half the crystal volume. Materials with an FD more than 12 contain rings consisting of 4 or more T-atoms (4-membered rings). The exception is lovdarite² which possesses

3-membered rings, some of which have T-atoms shared by 4-membered rings. Thus, Brunner and Meier designate it as 3+.

If the correlation between the minimum FD and ring size is correct, then zeolites with 3-membered rings must be synthesized to achieve materials with a higher capacity. Meier³ has offered several hypothetical structures which are based upon 3-membered ring building units with FDs in the range of 9–10. These very open networks are quite fascinating and lead one to speculate upon uses of such materials if they could be synthesized. One particularly important application could be the effective storage of methane.

Numerous hypothetical frameworks are



Hypothetical framework with large voids and three-dimensional extra-large pores.

given in the open literature (besides those in confidential documents) which stimulate the synthetic chemist. Until very recently, for example, the largest ring in zeolites or molecular sieves contained 12 T-atoms. But there are hypothetical frameworks with 18- and 24-membered rings^{4,5}. Synthetic strategies developed from these hypothesized ring structures led us to synthesize the first extra-large pore molecular sieve which contained 18-membered rings⁶.

One of the new frontiers in zeolite science is the synthesis of extra-large pore materials. The availability of zeolites with pores 1–2 nanometres across would open new catalytic chemistry in the petrochemical and life-sciences fields. The predictions of Brunner and Meier are particularly germane to this goal. The one known 18-membered ring structure has an FD of 14.2, and the hypothetical 24-membered ring structures have FDs below 12 (ref. 4). All of these frameworks contain 4-membered rings. Also, these networks possess one-dimensional channels which are circumscribed by the extra-large rings.

Meier³ speculates that all extra-large ring structures based upon 4-membered rings will have one-dimensional channel systems. This is because hypothetical frameworks with multidimensional extra-large channels yield FDs below 12. One-dimensional channel systems in existing zeolites do not produce particularly good catalysts: because reactant and product molecules have one way into and one way out of the structure, the molecule flow is extremely congested which leads to poor catalytic activity. On the other hand, if the structure contains pore systems with greater dimensionality, then the molecular traffic can be routed to enhance movement of reactants to the catalytic sites and removal of the products. Meier³ claims that a 16-membered ring framework can be derived using 3-membered ring units with FD = 9.3 (see figure). Thus, extra-large pore structures with multidimensional channel systems may be feasible.

The existence of lovdarite lends hope that frameworks based upon 3-membered rings can be synthesized. But because lovdarite contains beryllium, a highly toxic component, practical application of the concepts of Brunner and Meier rest on discovery of synthetic routes to 3-membered rings with elements other than beryllium. □

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Immunology

MHC meets mother's milk

Peter Parham

CLASS I major histocompatibility complex (MHC) glycoproteins are most familiar in the context of genetic polymorphism, transplant rejection and the presentation of antigen to T lymphocytes. These molecules bind intracellular peptides, carry them to the plasma membrane and thereby expose the cell's contents to cytotoxic T-cell surveillance^{1,2}. Infected cells are identified and destroyed by display of viral peptides, transplanted tissue through expression of foreign MHC. As well as the classical class I genes which encode the antigen-presenting molecules, there are many genes encoding proteins with distinctive sequences and patterns of cellular expression³. These non-classical genes vary between species and strains of mice: some are found in the MHC, others are not⁴ — one is even found in cytomegalovirus⁵. Despite extensive efforts, the functions of these genes remain a mystery. Confronted thus, with an expanding roster of non-classical class I genes and proteins earnestly seeking employment, it is refreshing to find a complementary approach incisively in action on page 184 of this issue⁶. Simister and Mostov have cloned complementary DNA encoding two subunits of an Fc receptor that is used by baby rats to salvage immunoglobulin G

from their mother's milk⁷ (see figure). Sequence analysis reveals a structure that undoubtedly puts this molecule, called FcRn, in the class I family and provides the first clear example of a biological function for a class I molecule that has nothing to do with T-cell recognition.

What makes FcRn a class I molecule? It consists of p51, a transmembrane glycoprotein, associated with β_2 -microglobulin; the p51 is organized into the characteristic domains of a class I heavy chain. These observations, together with the overall similarity and conservation of key residues in the amino-acid sequence, predict that the three-dimensional structure of FcRn will be similar to the classical class I protein HLA-A2 (ref. 1). But there are considerable differences between p51 and the heavy chains of antigen-presenting class I molecules. Out of 101 invariant positions in the extracellular domains (α_1 , α_2 and α_3) of class I sequences from six species⁸, only 44 are conserved in p51. These domains of p51 also have five deletions, one insertion and an approximate 25 per cent reduction in the number of charged residues, predominantly occurring in loops between strands of β -pleated sheet¹. These structural differences contain clues to the contrasting functions

of class I molecules that bind either to variable domains of T-cell receptors or to constant domains of immunoglobulins.

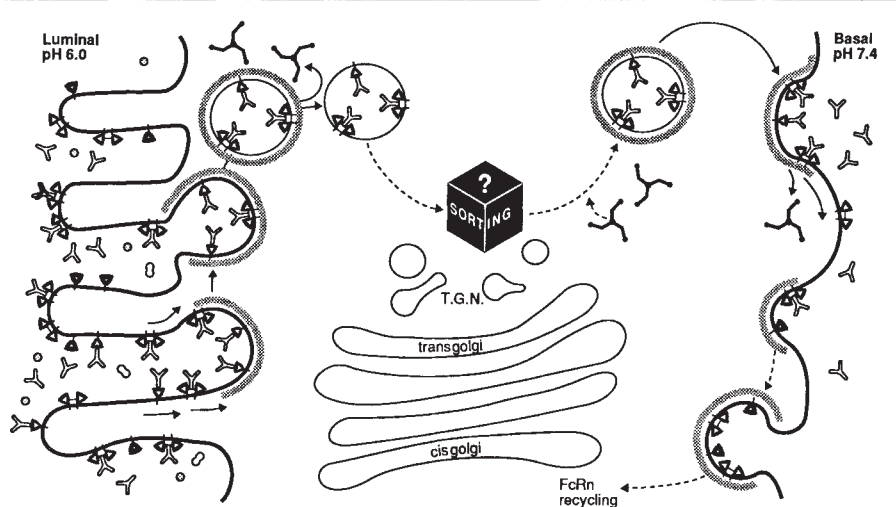
Binding of FcRn to IgG is tight at pH 6.0 and feeble at pH 7.4. Throughout their transcellular voyage, the FcRn and IgG complexes probably remain in acidic compartments as dissociation is reserved until arrival at the far side of the cell (see figure). In contrast, the antigen-presenting class I molecules bind tightly to their peptides at the 'physiological' pH (7.4) of cell surfaces, and it is this almost irreversibly formed complex that then engages in weak interactions with the CD8 glycoprotein and the antigen receptor of cytotoxic T cells. Although unsubstantiated by experimental evidence, the possibility is frequently raised that endocytic recycling through acidic intracellular compartments actually facilitates dissociation and exchange of bound peptides from classical class I molecules.

Raising the pH from 6.0 to 7.4 dramatically alters the interaction between FcRn and IgG. Histidine residues often titrate in this range and comparison of FcRn with classical class I sequences encourages suspicion of their importance for the pH-dependent binding of IgG. Of five invariant histidines (positions 3, 93, 188, 192 and 263) in antigen-presenting molecules, only that at position 263 is found in FcRn. On the other hand, FcRn has histidines at five positions (8, 171, 242, 255 and 256) where histidine is not found in classical class I sequences (see table).

FcRn has a structure distinct from other

Passive acquisition of immunity and an itinerant Fc receptor. One defining feature of the immune system is memory, others being specificity and versatility. An initial encounter with antigen generates the capacity for stronger and quicker responses on subsequent exposure to that same antigen. This property provides the rationale for vaccination against infectious agents. Newborn and young are particularly susceptible to infection as development of their immune system is incomplete and little is committed to immunological memory. To offset this vulnerability there are ways for mammalian young to benefit from their mothers' experience. In many species, maternal antibodies enter the fetal circulation *in utero* by crossing the placenta. For rats the passive acquisition of antibodies continues after birth but via a different route. Mother's milk provides the source of IgG which binds to a receptor (FcRn) of intestinal epithelial cells⁷. In a process called transecytosis, the IgG molecules are transported across the gut epithelium and can then enter the circulation¹⁵. Complexes of FcRn (black shapes) and IgG (white shapes) are internalized in clathrin-coated pits which are preferentially found at the base of microvilli that form the characteristic brush border of the luminal side of intestinal epithelium. FcRn is shown in both monomeric (single black shapes) and dimeric (double black shapes) forms. In common with systems of receptor-mediated endocytosis, the internalized pits form vesicles and tubules which lose their clathrin coat. Delivery to the basal surface may involve a second population of coated vesicles in a process perhaps similar to the sequestration of hormones, or lysosomal enzymes. The movements of this receptor do not involve the golgi cisternae⁷ but may involve transit through the trans-golgi network (TGN), a complex of membranous tubules and vesicles near the *trans*-face of the golgi which is thought to be a major intersection for intracellular traffic¹⁴.

FcRn, in common with other immunoglobulin receptors, binds to the Fc region formed by the second and third constant domains of the two IgG heavy chains. Its unique adaptation is that it binds IgG at the acidic pH (6.0) of the intestinal tract and releases IgG at the physiological pH (7.4) of the blood and tissue fluids⁷. These properties ensure unidirectional transport from gut to blood and were exploited in the affinity purification of this receptor on columns of IgG^{6,7,11}. This direction of transport is the opposite of that followed by the poly-Ig receptor in adult intestinal epithelium. In that pathway IgA produced by plasma cells of the gut associated lymphoid tissue is secreted into the intestinal lumen¹³. □



Histidines in class I molecules

Position	Amino acids found in:		Location based on HLA-A2 structure ^{1,2}
	FcRn	All antigen-presenting class I	
8	His	Phe	Interacts with β_2 -microglobulin and α_2
171	His	Tyr	Points into groove, interacts with α_1
242	His	Gln	Interacts with β_2 -microglobulin
255	His	Gln	Possibly close to CD8-binding site
256	His	Arg, Asn, Lys, Tyr, Ser	Possibly close to CD8-binding site, only Arg in humans
263	His	His	Common to immunoglobulin constant-like domains
3	Leu	His	On β strand of α_1
93	Phe	His	On β strand of α_2
188	Arg	His	Interacts with β_2 -microglobulin
192	Arg	His	Possibly close to CD8-binding site

Fc receptors, although all are members of the immunoglobulin superfamily⁹. Of great interest is the nature of its binding site for IgG and whether knowledge of the functional interactions of class I molecules in antigen presentation can help elucidate this question. One possibility is that Fc mimics the T-cell receptor, interacting with the helices of the α_1 and α_2 domains. In that case, does FcRn have the peptide-binding groove seen in the HLA-A2 structure^{1,2}, and if so does it contain a peptide — or perhaps some part of Fc? A second idea is that α_3 , the immunoglobulin domain of p51, interacts with Fc. Recent experiments suggest that the CD8 glycoprotein, another molecule with immunoglobulin-like domains, binds to the α_3 domain of classical class I molecules, probably to regions around residues 227 and 245¹⁰. FcRn has an accumulation of intriguing differences in this part of the molecule. The residue Ala 245, which is common to CD8-binding molecules, is changed to serine; position 228 has a glycosylation site; and residues 231 and 232 are deleted.

Simister and Rees have characterized¹¹ disulphide-bonded dimers of p51 and also inferred from electron irradiation experiments that the functional receptor is a dimer. A novel cysteine at position 230 in p51 might be instrumental in forming this dimer. Overall, this impressive set of structural innovations and the reconfiguration of histidine residues in α_3 favour this domain, possibly a pair of α_3 domains, as the binding site for an IgG molecule.

Jay Unkeless, a familiar figure in the Fc receptor field⁹, once remarked to me that a letter to *Nature* was the scientific equivalent of a birth announcement. In this instance he is probably right. Simister and Mostov have unexpectedly discovered¹² new links between areas of immunology, cell biology and reproductive biology. Kindled interest should lead this research in many directions: transcriptional analysis and transgenic mice (with healthier babies?) could help to reveal the precise expression in time and tissue of FcRn; transfection and mutagenesis to compare FcRn with the classical class I molecules of

the rat should yield insight into the structure and function of both molecules, and the way is solidly paved by Mostov's analysis of the poly-Ig receptor¹². The unusual cysteines, glycosylation sites and histidines of p51 all provide juicy targets for mutagenic mapping of the interaction with IgG. Furthermore, as Simister and Mostov point out, the cytoplasmic tail of FcRn is very different from that of antigen-presenting molecules, so tail-transplantation experiments should show if this sequence targets molecules to a pathway of transcytosis⁹. Comparison of FcRn and poly-Ig, two receptors that transport immunoglobulins in opposite directions across epithelia, raises its own questions. Why, for example, the perverse use of a receptor (poly-Ig) that is cleaved and destroyed after a single pass of the cell for extensive secretion of IgA in many tissues throughout adult life, whereas a function required for just a few

days in a single tissue uses a recyclable receptor (FcRn) that can transport multiple loads of IgG¹³?

The gene encoding p51 must also be mapped. Is it in the MHC, is it polymorphic, and is it specific to rats? A related question concerns the evolution of FcRn. Does this Fc receptor activity of class I molecules precede or proceed presentation of peptide antigens? T cells with their cell-surface recognition of peptides bound to MHC are probably of more ancient origin than B cells and soluble recognition of complex conformation by immunoglobulins. "Gin was mother's milk to her" — and milk, like gin, is a mammalian invention, whereas T cells and MHC molecules are also found in birds and amphibians. Such considerations, together with the restricted species distribution of FcRn, argue for immunoglobulin binding being a more recent adaptation of the class I molecule than antigen presentation. □

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Evolution

Anticipatory mutagenesis?

Neville Symonds

THE neo-darwinist position on evolution is that mutations occur at random and advantageous variants are favoured by selection. This orthodoxy was recently challenged by John Cairns *et al.* who, in a paper in *Nature*¹, proposed that favourable mutations can be specifically induced by the presence of a selective environment. This suggestion of directed mutagenesis, commented on at the time in the News and Views article² by Franklin Stahl, has stimulated considerable controversy, much of which has appeared in Scientific Correspondence (*Nature* **336**, 218 & 525; 1988, and see page 123 of this issue). Further fuel is certain to be added to the debate by the paper³ of Barry Hall in the December 1988 issue of *Genetics*.

In Hall's system, two independent mutations are required before bacteria can adapt to a novel environment. Neither mutation on its own is expected to confer

any adaptive advantage, yet in the presence of selection the double mutants occur at frequencies orders of magnitude higher than expected from the individual spontaneous rates. Somehow, the first mutation in the sequence is stimulated to occur at an exalted frequency even though it apparently confers no advantage in the selective environment. As Hall says, taken at face value, these results lead to ideas of anticipatory evolution, a very heterodox position indeed.

The genetic system used by Hall is the *bgl* operon, a cryptic (that is, usually non-expressed) set of genes in *Escherichia coli* which, when operative, allows cells to metabolize β -glucoside sugars such as salicin. The operon consists of three structural genes preceded by a short regulatory region which in the normal R⁰ configuration inhibits transcription. Mutations R⁰→R⁺ can occur which activate the operon. A *bgl*

derivative exists in which the *bglF* gene (encoding a protein necessary both to transport and phosphorylate salicin) contains an insertion sequence called IS103 (the IS are a class of transposons about 1 kilobase long which on insertion inhibit the expression of structural genes). In this derivative, activation of R^0 and excision of IS103 must occur before the strain can grow on salicin. MacConkey-salicin plates are used to identify Sal^+ revertants in Sal^- colonies. This medium contains enough peptides to allow colonies to grow to about 10^9 cells in 3 days, and a dye which responds to sugar fermentation. On these plates, Sal^- cells form white colonies and if any Sal^+ variants arise then, after a delay, red papillae appear on the surface.

Hall's investigation started with the observation that after a 2-week incubation more than 60 per cent of colonies of the Sal^- double mutant showed papillae on MacConkey-salicin plates. As the combined mutation rate of the two individual mutations in growing cells was about 10^{-20} , this high frequency was unexpected. What goes on in old colonies that is different from logarithmic growth? The key result of the paper is that most colonies of the double mutant, when sampled after 12 to 13 days (just when the first papillae are beginning to appear), contain between 10 and 50 per cent of Sal^- mutants from which IS103 has excised. This result argues strongly that excision of IS103 precedes the regulatory mutation in Sal^+ revertants. The surprisingly high proportion of excision mutants in the colonies (reminiscent of Shapiro's results⁴ on analogous transposon systems) indicates that a burst of excision activity occurs during late stages of colony growth. Does the high proportion of excision mutants reflect a specific or a general effect? And is it influenced by selection? Clear-cut results are reported, showing there is no increase in mutations to valine resistance within old colonies, and that no excision mutants can be detected in colonies on MacConkey plates without salicin. Also reported is an experiment, persuasive rather than convincing because of technical problems, indicating that Sal^- excision mutants have no selective advantage over double mutants during colony development.

Hall interprets his results as showing that in the period between 8 and 12 days up to 50 per cent of cells within colonies experience excision of IS103. The resulting mutants provide a population of cells sufficiently large that a replication-independent $R^0 \rightarrow R^+$ mutation occurs in one and generates a Sal^+ cell that grows by using salicin to form a papilla. The excisions occur only in medium containing salicin, despite the fact that the excision confers no selective advantage and serves only to create the potential for a secondary selectively advantageous mutation.

This interpretation is certainly thought-

provoking, but I think there are grounds for deferring acceptance of it at present. First, there is the difficulty of understanding how salicin, which supposedly cannot enter mutant cells, nonetheless influences excision. Second, there is a lack of controls on whether any other transposon activity is correlated with IS103 excision. And third is the feeling, arising from published studies on the *bgl* operon, that excision of IS103 should confer some selective advantage to cells in the presence of salicin. This is because changes in supercoiling, both positive and negative, are known⁵ to induce the cryptic *bgl* operon. Some alteration in DNA topology would seem to be a likely occurrence in cells comprising old colonies.

As an alternative to Hall's interpretation, an explanation based on orthodoxy is that physiological conditions in old colonies induce a burst of random transposition activity in a fraction of the cells. Some of these events lead to excision of IS103, and if salicin is present these cells have a selective advantage, eventually comprising a large proportion of the viable cells in the colony, and subsequently sporting the second mutation that leads to a Sal^+ revertant. In the absence of salicin, growth of all the cells remains restricted and the mutagenic effects of transposition eventually lead to cell death.

Hall's paper is undoubtedly a provocative contribution to the debate on the origin of mutants, and highlights two general points which have been alluded to by other contributors to the debate, but are worth stressing. First, some of the mutagenic systems which have been discussed refer to events promoted by transposons, whereas others refer to effects related to point mutations. These two classes of event are basically distinct, they have different molecular explanations and occur at different frequencies. It would seem prudent not to extrapolate from one system to the other. Second, the question of what goes on in old colonies under deprived conditions on agar plates, which is the essence of the present problem, will lead to an interesting liaison between the old and the new microbiology. It will mean a return to studies of impurities in media, cannibalization of dead cells, and even what constitutes cell death. Together with these considerations, it is necessary to find molecular answers to questions relating to local topology inside such cells, their osmotic properties and, crucially, the structure of mutants generated in these (to us) novel conditions. □

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Daedalus

Corporate strategy

LAST week Daedalus decided that the fat cells of the body are digital information-storage elements. A full cell is stable; so is an empty one. But a partially full one, even in perfect reaction equilibrium, is not. If its fat droplet begins to grow, it will become thermodynamically more stable, and will grow by further synthesis; let it shrink, and its increasing thermodynamic instability will shrink it ever faster until it is completely hydrolysed. Judging by the high metabolic turnover of our adipose tissues, this gain and loss of fat droplets is going on all the time. This makes no sense if fat is merely an inert fuel store, but is just what you would expect of an active data-processing system. The filling and emptying cells, says Daedalus, must be moving digits around in the manner of a 'bucket-brigade' delay line or magnetic-bubble memory.

Now a fat cell takes hours or days to switch between its full and empty digital states. Our fat cannot go in for quick-fire decision making; it must be entrusted with the basic, slowly evolving, instinctive aspects of our nature, like our body image and sense of identity. This agrees with popular stereotypes and the claimed formal distinction between 'fat' and 'thin' personalities, and the changes of character and emotional style often reported by those who have lost or gained significant amounts of fat. People who claim to feel their emotional certainties in their bones are slightly misplacing the source of the sensation!

To verify these bold speculations, DREADCO's biochemists are launching sharply focused, high-intensity ultrasonic beams into small areas of the fatty tissue of DREADCO volunteers. The idea is to set into resonant vibration those fat droplets which happen to be tuned exactly to the chosen frequency. Each droplet will soon fission, like an over-excited atomic nucleus, giving two smaller droplets. The thermodynamic doom which awaits small droplets will soon react them away completely. Thus the tiny area targeted by the ultrasonic beam will be sprayed with copious bit-errors. Adipose tissue, like the brain, probably uses a robust summation logic rather than a vulnerable binary code; even so, the local data should be well scrambled. The resulting psychological changes in the volunteers will take a day or so to emerge. By matching the site of each ultrasonic scrambling to its emotional effect, the DREADCO team should soon work out the exact distribution and nature of our fatty data. The way will then be open for a new psychoadipotherapy, healing and refreshing those stubborn personal obsessions and distorted self-images that conventional therapies cannot reach. David Jones

Birth of the D-E-A-D box

SIR—The recent isolation and characterization of eight genes in organisms ranging from *Escherichia coli* to man¹⁻⁸ leads us to define a new family of proteins. Based on their homology to the eukaryotic initiation factor, eIF-4A, and on biochemical data on some of the proteins, they seem to be involved in nucleic-acid unwinding. The proteins we describe here share some similarity with members of the superfamily of unwinding proteins described previously⁹⁻¹¹.

The figure shows an alignment of the primary structure of the eight proteins. Within the region similar to eIF-4A, the alignment reveals that 9.1% (37/408) of the amino acids are identical in all eight. An additional 16.2% (66/408) are conserved in seven of the eight proteins, or are similar throughout the family, according to the rules of Dayhoff *et al.*¹². The regions of high amino-acid conservation have similar spacings in all the proteins, suggesting that these sequences have simi-

lar or identical functions. The more divergent spacer regions may reflect functional differences between the proteins. The SrmB protein from *E. coli* is most distant from the others. Of the 27 amino acids which are conserved in seven of the eight proteins, SrmB differs in 15 positions from the amino acid present in all the other proteins, whereas Tif, PL10, p68, vasa, and MSS116 diverge from the other seven at only one, one, two, two and six positions, respectively.

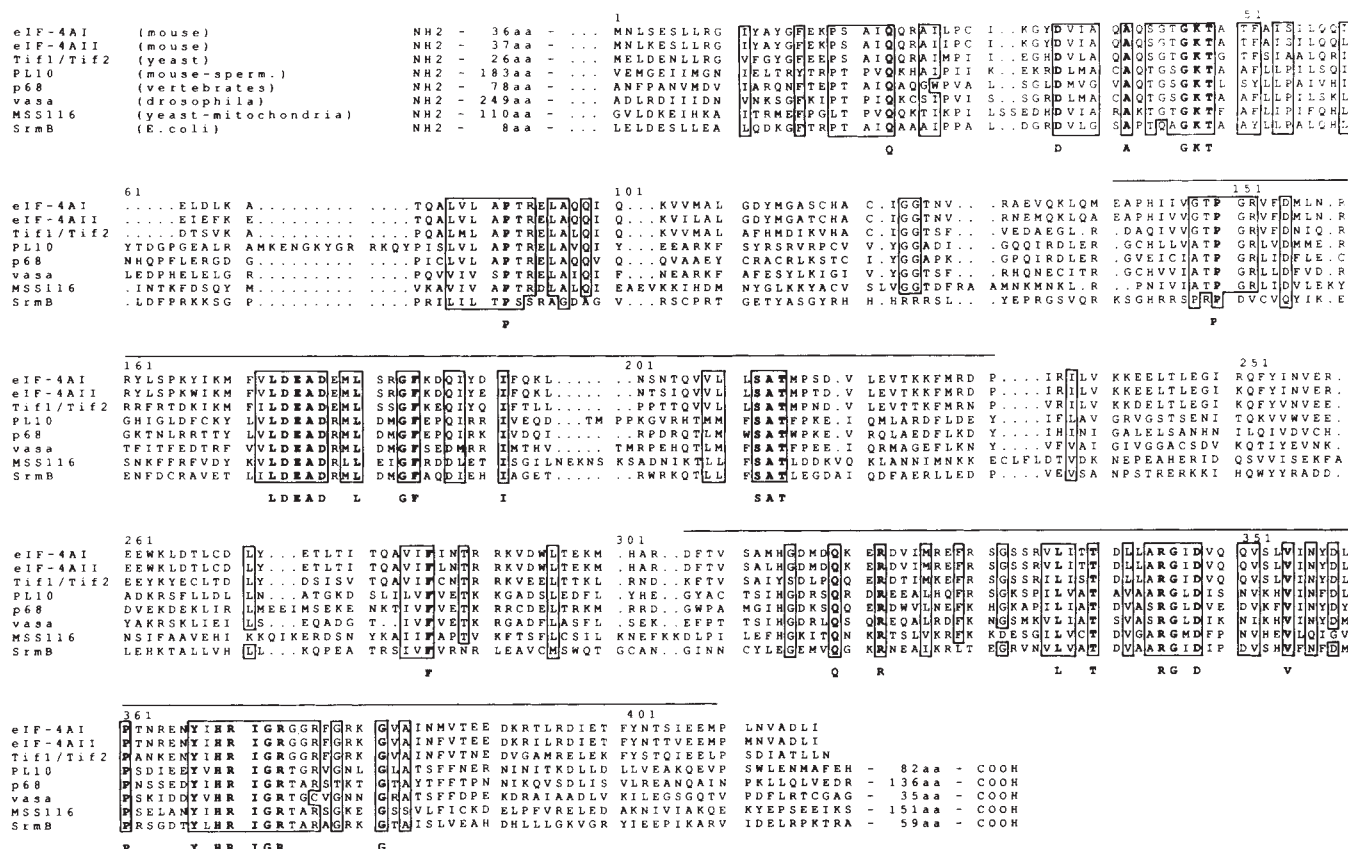
The sequence D-X-X-X-X-A-X-X-X-X-G-K-T, found in all eight proteins, is typical for the A-motif of ATP binding proteins^{9,10,13}. The presence of an alanine at position six instead of a glycine, as is found in most other ATP binding proteins, is noteworthy. Ford *et al.*⁵ report that in prokaryotic helicases (DnaB, RecB, Rho, UvrD) the glycine is also replaced by an alanine, and propose that this could be typical of helicases.

The D-E-A-D box, (V/I)-L-D-E-A-D-

X-(M/L)-L-X-X-G-F, represents a special version of the B motif of ATP-binding proteins¹³. The D-E pair is highly conserved in many DNA and RNA replication proteins^{9,10}. In a protein databank (MIPSOX, release 8) search, no other protein has been found to have the L-D-E-A-D-X-X-L motif, making this box characteristic of these proteins. Close to the D-E-A-D box, three other conserved amino acids, the S-A-T motif, are present. No function can yet be attributed to this motif.

A third region, of 73 amino acids with 35 conserved residues, is present between positions 311 and 383 of the aligned sequences (see figure). The most predominant consensus is the H-R-I-G-R motif. A search in the same protein databank for this motif revealed no further examples of it. It is tempting to attribute to this region a polynucleotide binding and/or unwinding activity, such as that proposed for the eIF-4A protein¹⁴.

In conclusion, we have defined a new family of proteins which all have several motifs in common. These motifs are prob-



The eight proteins have been aligned to demonstrate their similarities. Positions of similar or identical amino acids in the eight proteins are boxed, as are positions of 7/8 identities. Positions where all the proteins have the same amino acid are indicated below the sequence. The regions which show similar secondary structure predictions in a PEPLOT analysis¹⁵ are indicated by bars above the alignment. The sequences of eIF-4A1 and eIF-4AII are from mouse^{1,2}, Tif1/Tif2 are essential for viability and are thought to be the analogous initiation factor in *Saccharomyces cerevisiae*³, PL10 is a protein expressed specifically during spermatogenesis in mouse⁴, p68 is a major nuclear antigen from a dividing human hepatoma⁵, vasa is a protein from *Drosophila* involved in oogenesis and specification of the anterior-posterior axis of the embryo⁶. MSS116 is a protein from yeast involved in mitochondrial gene expression. Its gene was isolated by complementation of a nuclear *pet*⁻ mutation which affects splicing of mitochondrial transcripts⁷. SrmB is the product of an *E. coli* gene which, if present in high gene dosage, is able to suppress a temperature-sensitive mutation in the gene encoding the ribosomal protein L24 (ref. 8). The vasa sequence is slightly revised from that published in Lasko and Ashburner⁶. The differences are: position 230, N for L; position 231, Y for R; position 236, V for gap; position 246, A for R; position 384, T for Q.

ably involved in the ATP binding and ATPase activity and in the interaction with nucleic acids. Whether these proteins are homologous in the sense that they have diverged from a common ancestral protein, or have acquired their similarity by convergence, is unknown.

We would like to thank Bertrand Seraphin for communication of the MSS116 sequence and Reinhard Dölz for assistance with computer programs.

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Plant competition

SIR—Gaudet and Keddy¹ claim to provide a general non-phenomenological, predictive tool for studying competition in natural communities. However, there are several problems with their approach.

First, analysis of the experimental data is inadequate. After testing 44 species, Gaudet and Keddy found a strong relationship between the phytometer biomass and the traits of the test species, and a relationship of similar strength when a different phytometer (*Penthorum sedoides*) was used with a subset of ten test species. They also found just as strong a

relationship when they used traits of the test species grown in monoculture as predictors of the phytometer's biomass when it was grown with the test species. In all cases, however, Gaudet and Keddy failed to report or interpret the vector of multiple regression coefficients, which would indicate how, *as a group*, the traits define a good versus a bad competitor (for example, a good competitor has high above-ground biomass relative to below-ground biomass). These coefficients can be interpreted much like the coefficients of one of the extracted axes from a principal component analysis (PCA). The difference is that in PCA each axis defines a particular, independent trend in a group of variables, while in multiple regression the one extracted axis defines how a group of variables acts together in predicting a single, dependent variable. Thus, Gaudet and Keddy fall short of adequately describing the relationship between *Lythrum salicaria* and 44 competitors in a high-nutrient soil, and leave us with only biomass as a crude predictor of competitive ability.

More important, Gaudet and Keddy fail to demonstrate how their technique has "the potential for predicting the outcome of multispecies competitive interactions"¹. It seems reckless to assert that the ability to rank pairwise competition coefficients in a single set of carefully defined conditions will enable one to predict anything about higher-order interactions among species in a real multispecies setting. Even if one assumes no significant higher order interactions among species, the influence of habitat variation on pairwise competitive relationships must be considered.

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SIR—Gaudet and Keddy¹ claim to provide a tool for predicting the outcome of competition in multispecies communities. But there are problems with their study:

(1) The statistics used to demonstrate that there is a strong relationship between plant traits and competitive ability are invalid. This is because at the same time as the focal plant is being affected by its neighbours, the growth of the neighbours is being affected by the focal plant. The use of regression analysis is, therefore, not appropriate as the predictor variables are not independent of the response variables^{2–4}.

(2) The finding that interspecific ability is related to plant size is not surprising⁵ because they equate competitive ability with the reduction in biomass of the target phytometer. There are many precedents for this type of definition; indeed, we use it in our own papers^{4,6}. But such a definition gives particular weight to the effects

of plant size⁵ and by implication relative growth rate⁷. If this were the only factor determining the outcome of competition between species over several generations, then communities would be dominated by those plants with the highest relative growth rates. This is clearly not the case.

(3) The use of biomass reduction as an index of competitive effect is unhelpful in explaining the role of competition in determining the structure of natural communities. Here the emphasis should be upon the effects of competition on the per capita rate of increase, which is not perfectly correlated with biomass since it also depends on many other variables including population interactions such as herbivory and mutualism. It is simply not possible to forecast the outcome of competition over several generations on the basis of short-term changes in biomass⁸.

Gaudet and Keddy provide no indication of how their results can be used to predict community structure.

The search for general principles in competition studies has met with greater success than indicated by the authors. The effects of competition with monocultures and simple mixtures of plants is becoming well understood⁹; various plant strategies have been identified⁷; and the role of resources in determining the structure of communities has also been investigated¹⁰. But it remains difficult to predict the outcome of competition in a particular natural community, which is inevitable given that communities can be highly complex, non-linear multivariate systems which include variables that are by their nature unpredictable.

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SIR—Gaudet and Keddy argue¹ that the study of interspecific competition in community ecology has failed to yield any general principles. This conclusion is incorrect. The point is not that studies have "yielded an overwhelming body of special cases", but rather that they have shown that the outcome of interspecific competition is contingent on the specific conditions under which an experiment is conducted. In particular, the starting density and frequency of competitors can alter the outcome. Replacement series experiments often fail to produce general results because they do not explore the full range of densities needed to determine the trajectory a two-species mixture may follow¹¹.

The contingent nature of interspecific

competition is itself a fundamental principle and should not be mistaken for scientific imprecision. Gaudet and Keddy themselves over generalize from their experiments. It is hardly surprising that 40 species tested individually against a single 'indicator' species under uniform conditions can be ranked in their competitive ability on the basis of a single character. It is even less surprising that this character should be biomass. Repeating the exercise for 10 species with another indicator species does not improve the generality of these results because so many parameters important to the outcome of competition were left unaltered. More sophisticated competition experiments conducted along a nutrient gradient have shown that the competitive rank of a species in a mixture may alter along the gradient¹². Furthermore, even when competitive ability is fixed, its importance in determining a plant's distribution depends upon many other environmental variables (see, for example, ref. 13).

I agree with Gaudet and Keddy on the need for generalization in ecology, on the potential fruitfulness of the comparative approach when correctly applied, and on the importance of biomass in competition when other things are equal. But, oversimplifying experimental systems does not provide a short-cut to ecological generality or transform community ecology into a predictive science.

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GAUDET AND KEDDY REPLY—We demonstrated an empirical relationship between plant traits and interspecific competitive ability¹. To maximize generality, the study used 44 wetland plant species of widely contrasting ecology from across eastern North America. We suggested that such empirical approaches would be one tool for developing general predictive models for plant communities. We are encouraged that this paper has produced some lively debate, but believe that the criticisms are without foundation.

Firbank and Watkinson observe that the predictor variables (plant traits) were not independent of the response variable (phytometer biomass), but missed our point that when the analysis was repeated with predictor variables from monocultures, the same answer was found. Further, the phytometers were seedlings and it is likely that they had minimal effect upon the adult test species. Bailey's suggestion of a multivariate extension is a good one; we were aware of the possibility, but chose to emphasize simplicity in our paper.

Firbank and Watkinson assume that our competitive ranking by size translates into a measure of relative growth rates, but

there is no basis for this^{14,15}. We acknowledged that other traits besides size are important. Our technique is a tool for measuring (as opposed to speculating about) the relative importance of different traits in determining competitive ability.

Silvertown emphasizes the inherently contingent nature of plant competition. We offer, as an alternative hypothesis, that contingency is largely an artefact resulting from the examination of similar pairs of species. That is, once the relationship between size and competitive ability is eliminated, only residual variance (contingency) remains.

We wish to emphasize our long-term goal — general testable predictions about real multispecies communities — all three critiques of our paper focus on this point. Our paper presented some results allowing such predictions. This long-term goal has two components: (1) the ability to predict plant competitive ability, and (2) an understanding of the implications of different competitive abilities for community organization. Our results are consistent with evidence from plant strategies⁷. We also have pervasive evidence of competitive hierarchies in multispecies communities of wild plants and that position in these hierarchies can be predicted from plant size^{16,17}. Plant size is correlated with species distributions along natural environmental gradients¹³ and relative abundance in turf⁸. Our technique has therefore quantified a broad general principle regarding plant competition and interpretation of our results does not appear to be limited to the experimental conditions of our study. With respect to implications for community organization, we are currently testing whether our measure of competitive ability will predict field distributions in a field data set collected from wetlands in eastern Canada. A general model of centrifugal organization of communities has been proposed^{18,19}; an essential element of this model is size-related competitive ability for light.

We reiterate our view that the status quo is not producing rapid progress in the

study of plant competition at the community level. On of the major drawbacks of current pairwise analyses is that they restrict the number of species that can be evaluated in any experiment. In arguing that existing methods suffice, Firbank and Watkinson seem to confuse the ability to predict competitive outcomes of pairs of annuals grown in pots with the development of predictive models for natural multispecies communities. Further, asserting that a phenomenon (for example, resources) has been 'investigated' does not provide evidence of progress.

Finally, Bailey accuses us of being reckless. Our paper simply proposed a research strategy, documented a significant empirical relationship, and invited further work. Whether any research path will yield scientific progress is ultimately not a matter of debate, but a matter of time and testing.

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Another alternative to directed mutation

SIR—Cairns *et al.*¹ argue that bacteria can direct mutations, such that their rate of adaptation is enhanced. Their hypothesis rests on two types of observations. First, that the distribution of certain mutants obtained by selective plating from sister cultures is less variable than expected for spontaneous mutations. And second, following plating, certain mutants are observed after a delay that is inconsistent with their growth rate on the selective medium, implying that the mutations occur only (or more frequently) under selective conditions.

Alternative hypotheses have been

presented that could account for the unexpectedly low variance in numbers of mutants from sister cultures, most notably slower growth of mutants before selective plating^{2–5}. However, Cairns⁶ has challenged others to present alternatives to directed mutation that could explain why certain mutants appear only (or more frequently) under selective conditions.

We have such an alternative hypothesis. Consider the following haploid genotypes. A is used to found the sister cultures, and cannot grow at all after selective plating. A mutates to A', which grows slowly after selective plating. A' mutates

to *B*, which grows quickly on the selective plates. (For simplicity, assume that the rate of mutation from *A* to *B* is negligible.) We have investigated the properties of this two-step model, which we will present in more detail in a forthcoming paper. Under this model, many colonies are detected only after delays that appear inconsistent with the rate of growth of genotype *B* on the selective medium. Moreover, expected distributions of colonies obtained from sister cultures are similar to those presented by Cairns *et al.*¹. Thus, the two-step model accounts for both types of observation used to support directed mutation.

Although two-step mutations are easily overlooked, they have been demonstrated previously. For example, it has been known for some time that it is quite difficult to isolate mutants of *Escherichia coli* B that are resistant to phage T2 (refs 7, 8). Using the standard protocol of plating for resistant mutants in the presence of excess phage, one of us² calculated the mutation rate to be of the order of 10^{-10} per cell generation. When bacteria and phage were put into continuous culture, T2-resistant bacteria appeared much sooner than could be anticipated from this rate. This occurred because mutants that were partially resistant to the virus increased in frequency, and these partially resistant intermediates mutated to the completely resistant genotype at a rate about 100-fold higher than did the original sensitive genotype³. What appeared superficially to be an improbable event was instead a sequence of two unremarkable events.

Shapiro¹⁰, whose study of gene fusions generated by Mu excisions suggests directed mutation, observed that after cells were plated on selective medium, there eventually appeared "... numerous papillae on the background lawn. (Picking and testing of these papillae showed that some contained cells capable of full growth on minimal arabinose-lactose agar and some did not.)" Growth of cells other than those with the fully expressed mutant phenotype clearly indicates the potential for selective enrichment of an intermediate genotype and a secondary mutation. That the appearance of colonies was accelerated by exposure to arabinose¹ suggests to us that arabinose provides a substrate suitable for growth of some intermediate. Why might two mutations occur when apparently one suffices? It is

known that excisions of Mu are usually imprecise, so that two steps are often required to reactivate fully a gene inactivated by an insertion¹¹.

If enrichment of an intermediate genotype is demonstrated, it could still be argued (as Hall¹² has done) that it occurs not by selection, but rather by directed mutation from *A* to *A'* on the selective medium. Hall studied an adaptation that required two mutations — excision of an insertion sequence from a structural gene, which preceded a second mutation in a regulatory gene. Hall could detect no growth of the intermediate genotype on the selective medium, which is consistent with its directed mutation. However, Hall's Table 2 shows that the frequency of excision mutants varied greatly among sister populations on the selective medium

— after 12 days, for example, from less than about 1% to about 90% among six sister populations. This is not consistent with the random distribution expected under the hypothesis of directed mutation, unless one also invokes *ad hoc* variation among sister populations.

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● See the News and Views article by Neville Symonds on page 119 of this issue.

CO₂ from magma-chamber degassing

SIR—The high CO₂ concentration reported by Karl *et al.*¹ for hydrothermal vent fluids from Loihi Seamount underscores the need to place more emphasis on the role of magma-chamber degassing as a source of the CO₂ discharged at submarine hydrothermal vents. This source of CO₂ may be as important as, and possibly more important than, the customarily accepted source mechanism of hydrothermal stripping from sea-floor lavas and rocks of the oceanic crust. Although Karl *et al.* do not discuss the problem of source posed by the high CO₂ concentrations at Loihi, Cann and Strens do stress this problem in their News and Views article². One suggestion by Cann and Strens that comes close to the magma-chamber-degassing source for the origin of the high CO₂ is volcanic degassing, that is, degassing of magmas as they ascend and erupt onto the sea floor.

Studies of the Kilauea volcano, the sub-aerial volcano 50 km north-north-east of Loihi Seamount and the next oldest product of the Hawaiian hotspot, have shown that CO₂ degassing occurs continuously from its summit magma chamber^{3,4}. The CO₂ is derived from the basalt supplied to the summit chamber, and volatile budget models^{3,4} require that 90–97% of the CO₂ initially present in the parental magmas is lost in this way. Consequently, eruptions such as the current east rift zone eruption, which involve magma that has degassed previously in the summit chamber, produce volcanic gases that are greatly depleted in CO₂ (typically less than 5 mol %). Currently, summit magma-chamber degassing of CO₂ at Kilauea is about 30 times more effective than volcanic degassing of CO₂, even though an eruption is in progress. It would not be surprising if magma-chamber degassing similarly dominates volcanic degassing, as well as hydrothermal stripping, of CO₂ at Loihi.

Magma-chamber degassing of CO₂ may

also be an important source of the CO₂ discharged from the hydrothermal vents at mid-ocean ridges. The magmas supplied to ridges are probably poorer in CO₂ than those supplied to Loihi, but the extremely low solubility of CO₂ in mid-ocean-ridge basalt (MORB)⁵ and the ubiquitous presence of CO₂-rich vesicles in MORB lavas make magma-chamber degassing of CO₂ a plausible hypothesis for these sites as well. Although it may be less intensive at mid-ocean ridges than at hotspot volcanic centres such as Kilauea and Loihi, it could nevertheless be significant on a global scale, as magmatism at mid-ocean ridges comprises 75% of the Earth's current magma supply and has probably been the largest single contributor of magma to the crust for more than 2×10^9 years. Thus, magma-chamber degassing of CO₂ from MORB may be important in the transfer of carbon from the mantle to the surface reservoirs of the carbon geochemical cycle. CO₂ degassing from sub-ridge magma chambers may also provide a carrier gas for transferring rare gases and other mantle-derived volatiles to the ocean-atmosphere system and for fractionating carbon isotopes in the magma supplied to mid-ocean ridges. The $\delta^{13}\text{C}$ values of sea-floor MORB lavas that have experienced magma-chamber degassing would be low relative to those of their parental magmas because of the large positive fractionation factor for $^{13}\text{C}/^{12}\text{C}$ between CO₂ fluid and carbon dissolved in basaltic liquid⁶.

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The imperial theme

John Galloway

A History of the Imperial Cancer Research Fund 1902–1986. By Joan Austoker.
Oxford University Press: 1988. Pp. 375. £40, \$84.

RESEARCH institutes, like people, have secret lives. It is the job of a good historian, as of a biographer, to unveil them and suggest how they underlie the one on public show.

In this particular history, the contrast in willingness to display the secret and the more apparent lives is seen rather clearly, the result of what looks like cold feet on the part of the Imperial Cancer Research Fund (ICRF) and Oxford University Press. For this is not, as the title claims, a history of the Fund from 1902, when it was founded, until 1986. The history itself stops short in 1979, the year Sir Walter Bodmer became its scientific director, and from then on Sir Walter himself takes up the narrative in what is described, not very accurately, as an "epilogue". Dr Austoker seems to get close to the secret life in a readable piece of painstaking scientific history. Sir Walter's style is more that of an annual report to the shareholders — very much more the apparent life. There are shades here of all those writers, like T. S. Eliot, who refused to have biographies written about them. And who can blame them? But it has made this a less good book than it would otherwise have been.

We seek in the secret life insights into the apparent life. To pursue the literary biographical metaphor, what compulsions, rooted in the insecurities of its early life, led to features of the Fund's behaviour as it grew to adulthood?

Dr Austoker is skilled in tracing corporate neurosis. For example, she describes the successful libel action of July 1979 in which the High Court found for the Fund and against the *Sunday Mirror*.

The action was taken as a result of the newspaper's publication of claims that ICRF was investing too much of its income, and a clear implication that what people were giving was to pay for research, not to invest. Earlier that year *New Scientist* had also suggested that investment was far too big, as eight years previously had *The Lancet*. Even earlier, the Cancer Research Campaign had protested about a public appeal for funds by ICRF more or less on the grounds that the Fund already had enough money in the bank. The tendency to rely on or be defended by a large capital sum can be traced to the earliest days of the Fund's existence. Dr Austoker quotes from the first annual report of 1902, in which it was asserted that the aim was

... not to expend any part of the principal so that in the event of the nature and causes of cancer being discovered, the fund should be available for the utilisation and application of the new knowledge and prevention and curing the disease.

Someone clearly thought the war would be over by Christmas.

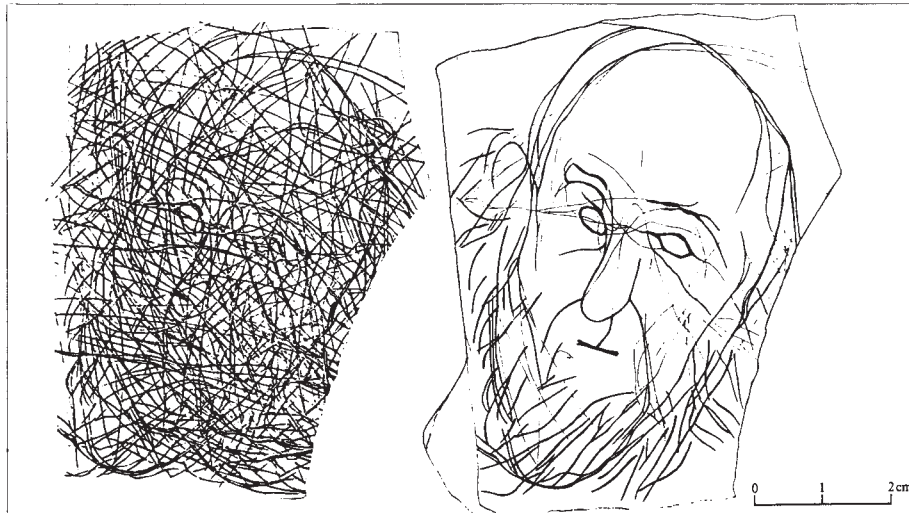
The run in with the *Sunday Mirror* was not the only legal action involving the Fund on a point of principle. Ernest Bashford, the Fund's first director, attacked quackery in cancer treatment in the *British Medical Journal* in 1911 and found himself on the wrong end of an action by Dr Robert Bell (whose patients were treated dietetically and with injections of formic acid). Science collided sharply with free-market medicine. And science lost. Bell was awarded £2,000 plus costs.

What characterizes ICRF and distin-

guishes it from the Cancer Research Campaign (for which I work), is that overwhelmingly it tends to support research in its own laboratories, most notably at those in Lincoln's Inn Fields, London. (By contrast, the Cancer Research Campaign supports research largely through grants to universities, teaching hospitals and a number of research institutes.) It is this commitment to a large permanent research structure that has, not unreasonably, been used to justify a continuing reliance on a large capital investment. The existence of a permanent research institute with a strong corporate identity — which ICRF certainly possesses — heightens our expectations of some sort of continuity in the themes of its research, not only innovation, which is important enough, but also a sustained interest and effort.

In practice it looks as though each new director tended to impose his own interests on the organization and to some extent sweep away what was there already. Yet although unbroken lines of research interest are not really traceable over long periods, the Fund's researchers have at different times made notable contributions to the development of important theories. Given ICRF's present-day commitment to dissecting the problem of cancer using molecular genetics, it is appropriate that Dr Austoker rehabilitates James Murray, the second director, giving him a good slice of the credit for the idea that cancers originate in chromosomal damage; Murray had been a student of Theodor Boveri before joining the Fund but it is usually Boveri who has the credit for these ideas early in the century. Murray was also able to demonstrate inherited tendencies to mammary cancers in mice through breeding experiments, important work, but work which could not be developed satisfactorily until much later when better strains of mice had been bred.

The Quaker pathologist, Cuthbert



Hidden treasure — the face of a bearded man painstakingly extracted from the muddled engraving on a plaquette found at La Marche, Vienne, France. The layer of the La Marche site containing these engravings, which is unusually rich in representations of the human form, is dated at about 14,280 years BP. The picture is one of many in Images of the Ice Age by Paul G. Bahn and Jean Vertut, a detailed examination of life in the Ice Age and of archaeological methods in general, accompanied by Vertut's colour photographs of European palaeolithic art. The book will be published on 26 January by Windward, London (price £15); Facts on File will be the publisher in the United States.

Dukes, who had a long association with the Fund, studied familial polyposis coli in the 1920s and 1930s, and seems to have come close to putting his finger on the idea that the development of a cancer requires several distinct genetic events. In an inherited cancer the first event is already present through the germ line, which Dukes and his collaborator Lockhart-Mummery did not perceive, and which was in fact suggested much later by A. G. Knudson working on retinoblastoma.

Tumour virology is an area in which the Fund has contributed signally to both of the branches involved — viruses as causes of cancer and viruses as means to understanding cancerous transformation of cells. William Gye, the Fund's third director, believed passionately in the tumour virus theory but it was not until a decade after his death that his belief was made manifest. Michael Stoker's appointment to the directorship in 1968 ushered in a decade of excellence in tumour virology, putting the Fund unmistakably at the forefront of international research.

Science, like pretty much everything else, is more interesting when its success or failure is seen in terms of the ambitions and weaknesses of those who pursue it. Is the history of a research organization more than the biographies of those who work for it? The rise and fall of scientific theories is dry stuff divorced from the rise and fall of those who create or espouse them. Poor James Murray, who became impaled on the discredited chronic irritation theory of cancer. And the life of his successor, William Gye, is the stuff of a rags to riches epic novel. Born in poverty, Gye left school at the age of 13 and worked as a stonemason and railway clerk, where he found time to study chemistry. Entering University College, Nottingham, he paid his examination fees by playing professional cricket. He read medicine at Edinburgh and later worked at the National Institute for Medical Research before becoming the director of ICRF. The previously esoteric and neglected viral theory of cancer gained greatly from his adoption of it.

It is not really surprising that much of the best part of Dr Austoker's history is 'cancer politics'. She deals adroitly with the dark days of the 1920s, when the Fund's great rival, the British Empire Cancer Campaign (later the Cancer Research Campaign), arrived to claim a share of what had until then been the Fund's territory; and with the attempt by Sir Walter Fletcher, secretary of the Medical Research Council, to establish the undisputed hegemony of the MRC in British medical research.

Why do we need books such as this? The answer is found in the words of Eric Larrabee:

The only thing wrong with scientists is that they don't understand science. They don't know

where their own institutions came from, what forces shaped and are still shaping them and they are wedded to an anti-historical way of thinking which threatens to deter them from ever finding out ["Science and the Common Reader", *Commentary*; June 1966].

Anything that tells scientists — and everyone else — about the origins of their national scientific institutions, how they work and how sometimes they don't, must be a good thing. When the account is as lucid, as scientifically well-informed and as thoroughly documented as this, it is a very good thing indeed.

It is therefore a pity that Dr Austoker was not allowed to finish her history. Her re-creation of the past should have been permitted to illuminate the Fund as it is today — we need to see it through an outsider's eye, and must always suspect the director of heroically justifying its existence. I cannot believe Dr Austoker did not do the research for the final part of the book. It ought to be published. □

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Physical interface

J. H. Mulvey

Nuclear and Particle Physics Source Book. Editor-in-chief Sybil P. Parker. McGraw-Hill: 1988. Pp. 529. \$35.

WHAT is a "source book", and who needs one? McGraw-Hill published a series of ten of them in 1988 containing, as they say, "up-to-date and comprehensive coverage" of topics as diverse as acoustics, meteorology, communications and physical chemistry, "to answer the need for targeted information on scientific and technological questions". The volume on nuclear and particle physics contains about 150 entries ranging in length from paragraphs defining a word to mini-monographs of over 20 pages. All are invited contributions from recognized authorities, including J. D. Bjorken on quantum electrodynamics (QED), the quantum field theory of electromagnetism, Alan Bromley on nuclear molecules, Dick Dalitz on baryons and mesons, C. N. Yang on gauge theory and Glenn Seaborg on transuranic elements.

Most of the articles are excellent, as one would expect of the authors, and many who work in these fields would find the book well worth dipping into. Their interest could be caught by Chris Quigg's readable essay on quantum chromodynamics (QCD), the theory believed to describe the strong force imprisoning quarks in the proton and neutron; or perhaps by Walter Greiner's account of super-critical electrostatic fields. These intense fields, capable of breaking down the normal vacuum — empty of real particles — into a new vacuum where real particles can exist, may occur in the near neighbourhood of two highly charged nuclei meeting in collision; there are analogies in the QCD mechanism that are proposed to be responsible for quark confinement and, in the case of gravity, the emission of particles from the vicinity of a black hole as predicted by Hawking. Although articles published in *Scientific American* are frequently cited in the

bibliographies, and many of the authors treat their topics at an equivalent level, some other entries make much greater demands on the reader's theoretical or technical understanding.

Such a book is not meant to be read from cover to cover, but doing so reveals more repetition than seems necessary. More seriously, there are some quite surprising omissions. There is no mention of nuclear magnetic resonance techniques as used in chemistry, biology and diagnostic medicine, yet NMR is one of the most powerful of the many tools passed on by physics to other sciences. In a book emphasizing the interface between nuclear and particle physics, it is odd that no place is found for the EMC (European muon collaboration) effect, dating from 1983, which directly probes the quark-gluon composition of nuclear matter — as opposed to that of free nucleons; nor is there adequate treatment of the many examples of cross-fertilization between particle physics and cosmology. The section on particle detectors is especially disappointing, perhaps reflecting, more than other areas, the origin of the entries in an encyclopaedia which is now in its sixth edition and which is apparently not keeping up with new experimental techniques. Inadequate cross-referencing, and some poor choices of section titles, will hinder the non-professional — the topic of stellar energy sources is not indexed, for example, and one has to know enough already to look under "proton-proton chain" and "carbon-nitrogen-oxygen cycles".

There are many meaty pieces in this hotch-potch of scientific nourishment but it would have benefited from tighter editing and better scientific guidance in its preparation. Whether such books are needed by the professional, given the wide range of up-to-date reviews available, is doubtful. But they do give an easily accessible overview of a wide range of topics, and students — and perhaps teachers — will find it useful to have them in the library for reference. □

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America's history in depth

John E. Pfeiffer

Ships and Shipwrecks of the Americas: A History Based on Underwater Archaeology. Edited by George F. Bass. *Thames & Hudson: 1988. Pp.273. £24.95. In the United States distributed by W.W. Norton, \$40.*

MANY great archaeological sites lie submerged, fathoms deep in places that have been inaccessible until quite recently. Archaeologists, having already recorded enough terrestrial sites to keep them and their students busy for a thousand lifetimes, now find themselves on the verge of a new wave of underwater discovery. The latest scuba gear is standard equipment, along with shovel, trowel, dental pick and other familiar excavating tools. In addition there is an impressive array of high-tech paraphernalia developed primarily for military reconnaissance and salvage operations (most notably the *Titanic* expedition of 1985). The list includes side-scan sonar, hand-held caesium magnetometers, manned mini-submersibles, and pilotless surveying and collecting vehicles guided by remote control.

This book views history from a subaqueous perspective, concentrating on New World shipwrecks in American rivers, lakes and coastal waters. A dozen nautical archaeologists describe some of their most memorable exploits, and look ahead to the future of their new discipline. Gordon Watts of East Carolina University was one of the team participating in the search for the Civil War ironclad *USS Monitor*, the "cheesebox on a raft" sunk in 1862 during a storm off Cape Hatteras, and rediscovered more than a century later with the aid of closed-circuit television and a computer-controlled positioning system. Watts recalls the "tremendous excitement" of his first dive in a submersible, seeing the upside-down wreck at a depth of 230 feet, and subsequent work outside the submersible using a hydraulic dredge to remove overburden and a special camera array to provide stereoscopic photographs of excavated areas.

Artefacts recovered from the *Monitor*, which was officially declared a National Marine Sanctuary, include an unusual four-fluked anchor, a brass distress-signal

lamp, and an assortment of bottles and storage jars, all of them on display at the Mariners' Museum in Newport News, Virginia. Kevin Crisman of Vermont's Basin Harbor Museum directed recent excavations of an earlier battleship, Britain's 16-gun sloop *Boscawen*, sunk at its moorings in Lake Champlain after successful engagements during the French and Indian War. Working in "extremely murky" waters, he and his co-workers managed to come up with thousands of items, everything from musket barrels and bayonets to hickory nuts, and seeds of pumpkins and wild grapes. Other investigators report on the continuing search for remains of Columbus's caravels (at least eight lost, none found to date), Spanish galleons, Basque whalers, Mississippi catamarans and side-wheelers, Yankee

to do with anthropology". That was a number of years ago, and unkind cuts from within the profession are generally less frequent and less biting than they once were. But the attitude persists in some quarters, and may influence hiring and tenure-track decisions.

All archaeologists stand together when it comes to looting, an activity that is perhaps a bit more blatant in the New World than in the Old, where tradition is sometimes effective in curbing or at least tempering greed. Bass points out that "all the known wrecks of the Age of Exploration were damaged by modern looters before archaeologists reached them". Plundering for profit continues by people who pay lip service to science and who, upon occasion, are abetted by the courts in the name of free enterprise. But we can look forward to better times ahead, to stricter law enforcement which will help preserve the most important sites for study and sight-seeing by schools of free-swimming, scuba-equipped tourists.

Perhaps the most pressing problem, now and in the long run, involves research strategies. Because only a small fraction of the tens of thousands of ships lost in American waters (some 6,000 are listed in official United States registers alone) can possibly be considered for excavation, site selection becomes crucial. Documenting anthropological universals, cultural patterns which seem to endure over the ages, can be as important and exciting as preserving historic vessels and associated artefacts. For example, the wrecks of Spanish galleons, eighteenth-century blockade runners, Great Lakes steamers, Liberty ships of the Second World War, and present-day supertankers attest to the dangers of building ships in haste for short wars and quick profits.

The horizons of underwater archaeology extend far beyond research on ships and shipwrecks. There are secrets beneath the seas, inundated hills and valleys where our prehistoric ancestors lived during times when ocean levels were several hundred feet lower than they are today. It is a safe bet that sooner or later deep-diving explorers will be digging sites on the world's continental shelves, using techniques and technologies put to the test by the contributors to this book. □

John E. Pfeiffer, PO Box 273, New Hope, Pennsylvania 18938, USA, is a freelance journalist and author of *The Creative Explosion: An Inquiry into the Origins of Art and Religion* (Harper & Row, 1982).



Sorry end — the Canadian steamboat Schwatka lies abandoned at an old shipyard on the Yukon river.

clippers and sundry ironclads that marked the end of the age of sail.

Underwater archaeology is a wide open field with its most significant advances yet to come. There are some problems, however. For one thing, landlubbing investigators have tended to look down on the pioneer efforts of their nautically minded brethren as essentially trivial. George Bass of Texas A&M University, editor of the volume under review, will not soon forget the response one of his grant proposals inspired in an anonymous referee: "Sounds like fun, but has nothing

From Ships and Shipwrecks of the Americas

Climate of change

Alan D. Hecht

Weather, Climate and Human Affairs: A Book of Essays and Other Papers. By Hubert H. Lamb. Routledge:1988. Pp.364. £60, \$99.

At a conference on "The Changing Atmosphere: Implications for Global Security", held in Toronto in June 1988, the prime minister of Canada, Brian Mulroney, and the prime minister of Norway, Gro Harlem Brundtland, called upon the international community to address the issues of global climate change, atmospheric pollution and their effects on society. These national leaders called for a "law of the air", or more broadly an international convention on climate.

The Toronto conference, and others that have taken place since then, demonstrate that the issue of greenhouse warming and climate change has expanded beyond the scientific community: it is now an issue for policy makers as well.

That climate is a factor to be considered in future social, economic and political planning would not surprise Professor Hubert Lamb, a pioneer in the study of natural climate change and its effects on society. Throughout his 30 years of pro-

fessional meteorological service, Lamb has done much to advance the fundamentals of meteorology and its links to the humanities.

Weather, Climate and Human Affairs contains 18 essays, some new, others originally published over the past 15 years but updated for this volume. In his introduction, Lamb explains the rationale for compiling the book: "Now that the study of climate and its development has emerged from long years of neglect, this relevance [of climate change to human affairs] is likely to be more widely appreciated than it was when the earlier papers in this book first appeared. Not all the items included here have been published before, and those that have appeared before were found in an inconveniently wide variety of sources".

This is not a technical book for research climatologists or meteorologists. Instead it is directed to a broader audience of scientists, environmentalists, and political and social planners who need to be reminded of the natural variability of climate, and who must address the problem of planning for future climate changes whether they are natural or man-induced.

I found most of the pieces on historical climatology (Chapters 1-12) a refreshing reminder of how human development is linked to climate change, and of how often historians and economic planners have ignored the environment as a factor in social and economic events. However, the remaining essays on climate change have weaknesses — Lamb's failure to make clear that the concerns over climate change have gone beyond carbon dioxide to a whole suite of greenhouse gases, including the chlorofluorocarbons and methane, means that many of the essays are out of date.

Lamb has also failed to update some of the essays adequately, or place others in proper perspective. Chapters 2 and 18 are good examples. In the article written for *Encyclopedia Britannica* in 1975 (Chapter 2), he writes that "it gives an introductory survey of the development of climate over the Earth from the last ice age to today". He concludes that "at present time natural fluctuations are still the dominant element in the Earth's climate system".

This may well be true, but detection of man-induced climate change is a hotly debated subject. Average global temperatures have been steadily rising for the past two decades. Four out of the past ten years have turned out to be the warmest in 150 years, and it looks as if 1988 will see a continuation of this upward trend. Long-term changes in precipitation across different latitudes are also consistent with model projections for greenhouse-induced climate change. Scientists are keenly arguing whether the greenhouse effect is reflected in these data, yet

Lamb's essays do not convey to the inexperienced reader the excitement of the contemporary debate.

Lamb himself is cautious in accepting evidence for greenhouse warming. In the acknowledgements, he asks his friends and colleagues not to be "dismayed by my limited acceptance of the fashionable carbon dioxide warming thesis, which looks so straightforward in the physics department but about which I believe there may be reasons for reservation in the wide world of atmosphere, oceans, soil and biosphere". In Chapter 18 he adds that "There are signs that the eager search in many laboratories for verification of the expected carbon dioxide caused warming of the climate may have been pressed too hard". And while he suggests that the increasing levels of carbon dioxide in the Earth's atmosphere should raise the world's temperature, he says the "effect may be smaller, perhaps very much smaller than is usually supposed". He even leaves open the prospect that a natural cooling trend, a return to ice age conditions, may balance man-induced global warming. Why such scepticism?

It is clear that Lamb is thinking about the role feedback plays in the climate system. There are indeed large uncertainties about the consequences of changes in snow and ice albedo and clouds in climate models. The ocean itself may delay or ameliorate the expected warming effect. But nowhere does Lamb deal with this subject, nor does he mention any possible positive feedbacks in the climate system which may result from the increased concentrations of methane in the atmosphere.

From the book it is clear that man has always lived with and adapted to climate change. Such lessons offer an opportunity for the public and governments to reflect on future adaptations and the implications of current societal goals and values. What is new is that mankind is now altering the environment in ways not previously imagined. Human-induced climate change is a global problem and will have to be addressed, in one way or another, by the international community.

As a measure of how fast the subject is developing, and how rapid has been the growth of political awareness, contrast the events described in the opening paragraph of this review with the concluding statement of the final chapter in Lamb's book: "At the time of writing it seems doubtful whether our politicians are as aware as the general public of the dangers and responsibilities implied [from climate change]". That, happily, seems no longer to be the case. □

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The recent excitement about neural networks

Francis Crick

The remarkable properties of some recent computer algorithms for neural networks seemed to promise a fresh approach to understanding the computational properties of the brain. Unfortunately most of these neural nets are unrealistic in important respects.

THERE has been a lot of excitement recently about neural nets. A new algorithm has produced quite simple nets that perform surprisingly well. A thick two-volumed work, *Parallel Distributed Processing*¹, has been a best-seller, read enthusiastically by psychologists, computer designers and physicists. Even undergraduates are now designing new networks. The interested spectator may well wonder what it's all about. What are neural nets? How do they work? And what, if anything, do they tell us about the brain?

Neural nets are composed of 'units' that have some of the properties of real neurons². That is, each unit has many inputs, some of which excite it and some of which inhibit it. The unit usually takes a weighted sum of all the inputs and puts out a single output, down the 'axon', if the weighted sum exceeds some threshold. In very simple nets the output may have only two states (0 for inactive, 1 for active). Other nets may use units with graded output, in which the output scalar can be loosely thought of as representing the average spike rate of a neuron. Such parallel nets are not, in most cases, built using analogue components. Instead they are usually simulated, rather laboriously, on a computer.

Everyone would agree that to understand the brain we must know how groups of neurons interact. As we are sure that many synapses are plastic, that is, they can change their strength with experience, it is also important to know the abstract rules imposed on such changes by the intricate biochemistry of a neuron. Thus a net is characterized by the properties of the units that make it up, the way they are connected together, and the algorithms used to change the strength of those connections.

Memory

What have we learnt from such theoretical models? An early discovery was that memory could be stored in a way very different from memory storage in a standard digital computer (see ref. 3 for a recent historical review). This is perhaps not too surprising. A typical computer is made with very fast components, each

having rather few inputs and capable of sending a pulse-coded message. Part of each message is the 'address' that indicates where a particular memory can be stored, and part is the information to be stored. The operation of the computer is largely serial.

The brain is different in almost every respect. Neurons are slow, operating in the millisecond time range, and typically have many hundreds or thousands of inputs. Although many of them produce action potentials or 'spikes' whose distribution in time is not completely random, there is no obvious sign of precise pulse-coded messages. Moreover the parts of the brain seem to be highly parallel in their operation⁴. How then can an assembly of neurons—a net—store memory?

Notice that there are three aspects of processing a memory: putting it into the net, storing it there over time and retrieving it when required. Leaving aside immediate or working memory, which may be rather different, it is widely believed that the first and third operations (the in and the out) require neural activity, whereas the second one—the long-term storage—does not. The memory, so the gospel goes, is embedded in the strength of the numerous connections or synapses in the network. Most neural nets also have this character.

What has to be stored in a net is the capacity to produce a particular pattern of activity in a group of units. By suitably adjusting the strengths of all the synapses, using a simple, local rule, a net can produce a pattern, given a suitable 'clue'. The clue can be any smallish part of the desired pattern. This can be done especially easily if the net feeds back on itself. Given a part of the input pattern, the net, by self-excitation, will regenerate the whole pattern. Such a system is called content-addressable, because any part of the pattern can act on the clue, which provides the address. Moreover, nets of a reasonable size can store several patterns. If they are sufficiently distinct the patterns will not interfere with each other. Thus the system is distributed as one memory is distributed over many synapses; superimposed, because one synapse can be involved in several memories; and robust, because

altering a few synapses degrades the performance very little⁵.

Such nets are usually simple, having a single layer of units. Moreover they are usually unsupervised. That is, there is no teacher to tell the net how to adjust its output to make it resemble the desired one. The net learns by using an algorithm based on an idea of Hebb⁶. This is a local algorithm as it depends only on the activity near that particular synapse: roughly, the synapse is strengthened if it receives an input signal on the presynaptic side, together with some indication of activity, such as the unit firing, on the postsynaptic side.

Back propagation

Memory, however, is not the only thing the brain needs to achieve. Another type of useful net is one that can extract categories. That is, it must search for regularities and correlations in the incoming signals and try to embody them in some way in its performance. It turns out that a single layer of units without feedback has severe limitations³. Even if each unit is told after each trial whether it should have fired faster or slower, a procedure known as supervised learning, it cannot be trained to perform even quite simple operations. The classic example is the exclusive OR (A, or B, but not both A and B). This can easily be done if a net of several layers is allowed. Unfortunately this leads to a problem: of all the various synapses, which ones should be adjusted to improve performance? This is especially acute if the synapses lie on several different layers of neurons.

The recent excitement has sprung mainly from a neat algorithm which solves this problem surprisingly well⁷. The full name of the algorithm is 'the back propagation of errors' but it is often called backprop for short. It can be applied to any number of layers, although only three layers are usually used: an input layer, a middle layer (referred to as the hidden units) and an output layer. A unit in each of the first two layers connects to all units in the layer immediately above (Fig. 1). There are no reverse connections or sideways connections—a simple net indeed. Each unit forms the usual weighted sum

of its inputs and emits a graded output.

The net works as follows. It is first set up with random connections. A particular input is then given to it, which produces activity in the output layer. A teacher, who knows what the response of each output unit should be for that particular input, indicates to each unit the size and sign of its error. For a theoretical model, the teacher is usually the person designing the net. In the brain the teacher is presumed to be another part of the brain. The error signals are used to adjust the weights of all the connections to the top or output layer and this information is 'back-propagated' to the hidden units in the middle layer. They use this information to adjust their synapses, which come from the axons of the input units. The exact way this is done conforms to common sense, but non-mathematicians often find the algebra rather daunting, so I will not attempt to describe it here⁷.

It can be shown that, in effect, these adjustments are equivalent to a method of gradient descent⁷. This means that the algorithm used makes a small adjustment to the strength of each synapse, in such a way that each alteration reduces the total error in the performance of the net. Applied repeatedly, this necessarily leads to a minimum in the total error. It is well known that such methods run the risk of being trapped in a local minimum⁷, which may be far above the global minimum. But in these nets this rarely happens, probably because of the many units involved and because of their graded response. Moreover the absolute global minimum is usually not required, only a sufficiently deep one.

NETtalk

The results that can be achieved with such simple nets are astonishing. A striking example, due to Sejnowski and Rosenberg⁸, is called NETtalk, whose task is to learn to pronounce English. It takes an English text as its input (Fig. 2) and its output is fed to a machine that can produce voice sounds. At first, having only random connections, it babbles, but gradually, as training proceeds, it starts to speak more intelligibly. Eventually, when tested with a text it has never seen before, it produces quite passable English speech, with about 90% accuracy (it could never be 100% correct because pronunciation depends somewhat on context in English, and the network knows nothing of meaning). Thus it has learned the rules of English pronunciation, which are notoriously not straightforward, in a tacit manner, from examples only, and not because the rules have been explicitly embodied in some program.

How does it do this? It is relatively easy, once such a net has been trained, to examine the 'receptive fields' of all the units in the hidden layer (by 'receptive'

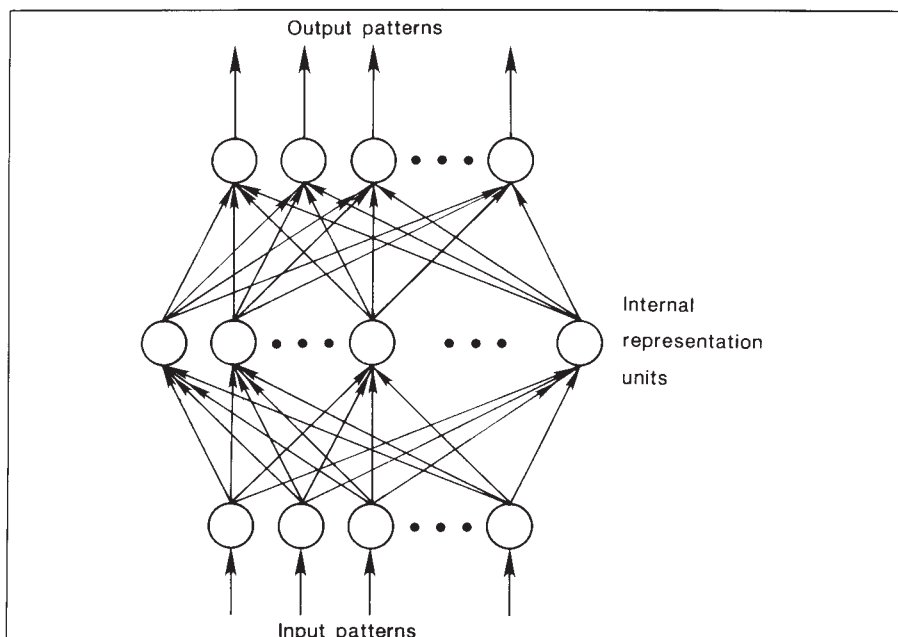


Fig. 1 A multilayer network. The internal representation units are sometimes termed hidden units. The information coming to the input units is recoded into an internal representation and the outputs are generated by the internal representation rather than by the original pattern. Input patterns can always be encoded, if there are enough hidden units, in a form such that the appropriate output pattern can be generated from any input pattern. (Reproduced, with permission, from ref. 1).

field is meant those features of the environment to which the unit responds). The results are remarkable⁸. The required information about categories is distributed across these neurons, because all information has to pass through the hidden layer, but not by any means in a random fashion. The hidden units have latched on to significant aspects of English speech, such as the difference between vowels and consonants, and indeed the different subclasses of these categories.

Further studies⁸ have shown that it is important to have the correct number of hidden units. With too few, the net simply cannot do the job. Too many, and, although the net works a little better, it does not generalize as well—it fails on English text it has not been trained on. It has become, as it were, merely a look-up table. But given the correct number of hidden units it will extract significant categories, which it can use successfully on untested material of the same general kind.

This is not the only example. Other striking applications are to the problem of deducing the secondary structure of a protein from its amino-acid sequence⁹, the distinction between rocks and unmentionable metal objects on the sea bottom¹⁰ and the derivation of shape from shading¹¹.

The last example also teaches us a further lesson. The units produced in the shape-from-shading problem turn out to be rather like edge or line detectors in the visual cortex. This alerts us to the fact that the receptive field of a neuron, by itself, does not necessarily tell us what its main function is. This also will depend on where

such a neuron projects, its 'projective field' as it has been called¹¹.

Neural nets and the brain

It is hardly surprising that such achievements have produced a heady sense of euphoria. But is this what the brain actually does? Alas, the back-drop nets are unrealistic in almost every respect, as indeed some of their inventors have admitted. They usually violate the rule that the outputs of a single neuron, at least in the neocortex, are either excitatory synapses or inhibitory ones, but not both¹². It is also extremely difficult to see how neurons would implement the back-prop algorithm. Taken at its face value this seems to require the rapid transmission of information backwards along the axon, that is, antidromically from each of its synapses. It seems highly unlikely that this actually happens in the brain. Attempts to make more realistic nets to do this¹³, though ingenious, seem to me to be very forced. Moreover the theorists working on

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the subject are so remote from actual neurons that they have been cavalier in omitting one type of unit altogether. Obviously there should be a unit to compare the output of each output neuron with the signals from the teacher, in order to calculate the error (in back-prop nets this is done by the computer). Such a set of neurons, if they exist, should have novel properties and would be worth looking for, but there is no sign of back-prop advocates clamouring at the doors of neuroscientists, begging them to search for such neurons.

There are many return pathways in the brain⁴, but we do not yet know if any of them act as one of the proposed teachers. Notice that to send separate teaching signals to each output neuron, a pathway must carry a lot of detailed information. We do indeed see diffuse pathways, such as that from the locus coeruleus, but one such neuron sends much the same signal to many parts of the brain, so that the information it can convey is somewhat limited and certainly would not be enough to control back-propagation. It may of course be used to tell the system when something is worth remembering. Models along these lines have been suggested, for example by Barto⁸.

Another problem is that though the back-prop algorithm can be generalized to a system with several successive hidden layers, it becomes extremely cumbersome. An ingenious way round this, suggested by Hinton¹⁵, is to train the net to make its output exactly the same as its input. This still allows the hidden layer to extract categories. In such a system the true output is taken directly from this hidden layer and made the input for the next set of nets. This allows any number of nets to be stacked on top of each other. If this were combined with a diffuse signal to indicate that something worth remembering has occurred, it begins to have some faint resemblance to what we see in the brain, but we are still stuck with the problem of how to implement back propagation realistically. Obviously what is really required is a brain-like algorithm which produces results of the same general character as back propagation. Another objection is that the back-prop algorithm is too slow, though it is not easy to make this argument

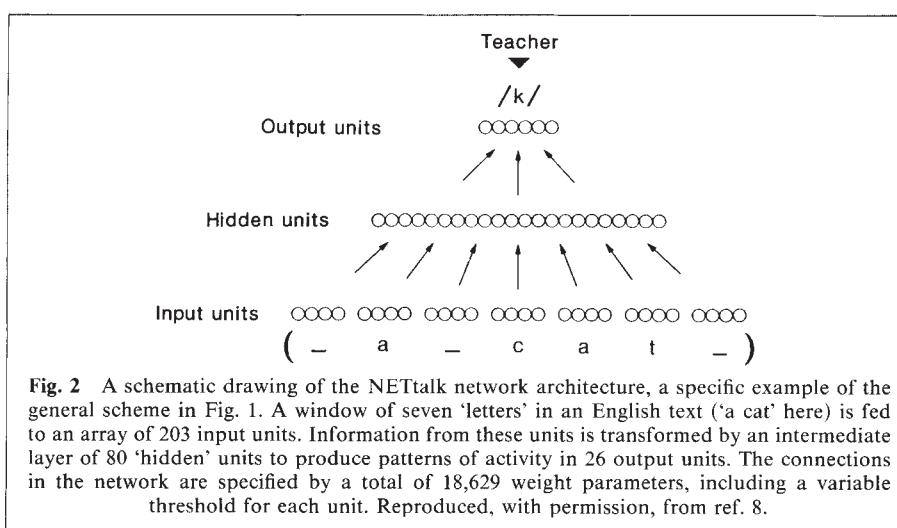


Fig. 2 A schematic drawing of the NETalk network architecture, a specific example of the general scheme in Fig. 1. A window of seven 'letters' in an English text ('a cat' here) is fed to an array of 203 input units. Information from these units is transformed by an intermediate layer of 80 'hidden' units to produce patterns of activity in 26 output units. The connections in the network are specified by a total of 18,629 weight parameters, including a variable threshold for each unit. Reproduced, with permission, from ref. 8.

quantitative in a realistic way. In any case modifications are now being suggested which can work rather faster¹⁶.

Most of these neural 'models' are not therefore really models at all, because they do not correspond sufficiently closely to the real thing. I have suggested elsewhere¹⁷ that they be called 'demonstrations'. They refute the claim that it is impossible for any neural net to act in such-and-such a way, but they may not perform in just the way the brain does. In another context they might reasonably be referred to as existence proofs. As such they have a certain use. The back-propagation algorithm can be used to generate a set of useful synaptic weights. This may not be the way the brain arrives at them—it may use some other more realistic algorithm—but the resulting receptive fields may, in their general character, be somewhat similar to those the brain has arrived at. This can be tested experimentally.

A good example of this approach is the work of Zipser and Andersen¹⁸, modelling a subset of posterior parietal neurons in the macaque monkey. Another promising case is the modelling of the vestibulo-ocular reflex¹⁹. In both, the character of the hidden units produced by back propagation is somewhat similar to what is found in electrophysiological recordings. Nevertheless, as far as the learning process is concerned, it is unlikely that the brain actually uses back propagation. In spite

of this, back propagation has been greeted with widespread enthusiasm.

Why the excitement?

How has this curious situation arisen? Apart from a few enthusiasts, most theorists do not believe that, for example, children really learn to speak using a single, simple back-prop network inside their heads. Why, then, are such models considered not only useful, but exciting?

To understand this we have to look at the structure and history of the various disciplines involved. It comes as a surprise to neuroscientists to discover that many psychologists, linguists in particular, have very little or no interest in the actual brain, or at least what goes on inside it. The brain, they feel, is far too complicated to understand. Far better to produce simple models which can do the job in an intelligible manner. That such models may have little resemblance to the way the brain actually behaves is not seen as a serious criticism. If it describes, in a succinct way, some of the psychological data, what can be wrong with that? Notice, however, that by using such arguments, one could easily make a good case for alchemy or for the existence of phlogiston.

The position is complicated by the fact that there is another application for network models: to assist in the design of novel, highly parallel computers. For this it makes no difference how the brain works and it is in this general area that most advances are now being made. Eventually, of course, the computer circuitry will have to be embodied in some sort of chip, which will bring its own design problems. The back-prop algorithm can be used to develop a good set of weights for special purpose chips, though eventually more versatile chips will be needed, with modifiable connections. Meanwhile, so the argument goes, why not develop networks and algorithms to see which systems perform best. With luck this may give theorists some experience of how complex

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non-linear nets behave in practice. Whether this experience can usefully be applied to models of the brain remains to be seen.

I also suspect that within most modellers a frustrated mathematician is trying to unfold his wings. It is not enough to make something that works. How much better if it can be shown to embody some powerful general principle for handling information, expressible in a deep mathematical form, if only to give an air of intellectual respectability to an otherwise rather low-brow enterprise.

At the bottom, one has to realize that these various activities, though superficially similar, are of a radically different kind. Constructing a machine that works (such as a highly parallel computer) is an engineering problem. Engineering is often based on science but its aim is different. A successful piece of engineering is a machine which does something useful. Understanding the brain, on the other hand, is a scientific problem. The brain is given to us, the product of a long evolution. We do not want to know how it might work but how it actually does work. This has been called 'reverse engineering'—trying to unscramble what someone else has made—but as has been pointed out, it is reverse engineering²⁰ on the products of an alien technology²¹.

And what a technology! Natural selection is not a clean designer. As François Jacob²² has pointed out, evolution is a tinkerer. It has, broadly speaking, to build on what went before. It can take a simple, straightforward process, such as DNA replication, and embroider it with any amount of gadgetry to make it work a little better. It is opportunistic: anything will do as long as it works. Naturally it is constrained by both chemistry and physics, but this does not necessarily mean that its mechanism will embody deep general principles; the structure of the genetic code is a good example of this. It may prefer a series of slick tricks to achieve its aim²³. Only a close inspection of the gadgetry will tell.

And this brings us to the crux of the matter. Why not look inside the brain, both to get new ideas and to test existing ones? The usual answer given by psychologists is that the details of the brain are so horrendously complicated that no good will come of cramming one's head with that sort of information. To which the obvious reply is, "If it's as complicated as that, how do you hope to unscramble its workings by a purely black-box approach, by merely looking at its inputs and outputs?"

By looking inside the brain we now strongly suspect that in important cases, at least in vertebrates' synaptic modification depends on the behavior of the NMDA-type glutamate receptor (see, for example, various articles in ref. 24).

This receptor works on a slightly slower timescale than the other glutamate receptors. It will open only if it has received the neurotransmitter glutamate, or something like it, during the recent past, provided the local negative voltage of the cell membrane has become somewhat more positive than the normal resting potential as a result of other inputs. When it does open, it lets in a lot of calcium ions, thought to be one of the initial signals in the complicated process of synaptic modification. It is thus ideally suited to perform associative learning.

Models embodying behaviour of the NMDA receptor would be most welcome and indeed such work is already in progress (D. E. Rumelhart, personal communication). As the weight of an NMDA receptor depends on the activity of neighbouring synapses in altering the mem-

brane potential, this opens the possibility of multiplicative interactions between synapses. We urgently need to know the exact location of NMDA receptors on neurons of all types and also the origin of the axons from which they receive glutamate. Learning about neurons, their behaviour and their connections will not by itself solve our problems, but will at least suggest the sort of answers to look for and can be used, often rather decisively, to disprove false theories.

Thanks are due to Patricia Churchland, David Rumelhart and Terry Sejnowski for helpful comments. This work was supported by Kieckhefer Foundation and the System Development Foundation. □

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Acknowledgements should be brief. Grant numbers and contribution numbers are not allowed.

Chaotic streamlines in pre-turbulent states

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Various patterns arise in hydrodynamic systems during the approach to turbulence. Under a wide range of conditions, these patterns have a symmetry typical of crystals or quasicrystals. Pattern elements (cells) are divided by thin layers which form a web, within which the streamlines are chaotic. The web represents channels along which particle transport occurs in lattice structures that are associated with dynamical chaos.

OUR understanding of the transition from laminar to turbulent motion is still far from complete. Clarification of the role of dynamical chaos as an important feature of turbulence has allowed a considerable advance in this field. The onset of turbulence is accompanied by random time variations of the velocity field (temporal chaos). An equally important property of turbulence is the spatially randomized structure of the velocity field (spatial chaos). A combination of these two fundamental properties of turbulence is beyond the reach of current theory. It is known, however that the formation of ordered structure may precede the onset of turbulence. Such structures are exemplified by the square (or rhombic) and hexagonal cells of Rayleigh-Bénard convection, and have been observed, for example, in rain clouds, in zonal flows in planetary atmospheres (of the Earth, Venus and Jupiter) and in thin foam films on the surface of solids melted by high-intensity laser pulses. Different environments give rise to differing stable patterns.

The different structures that arise can be considered as manifestations of a certain symmetry that is characteristic of an invariant property of the dynamical system¹. A non-trivial realization of this approach is given by Chernikov *et al.*², who consider the generator, M_q , of the plane tiling with q -fold quasicrystal symmetry. The values $q = 4$ and $q = 3, 6$ correspond to periodic tilings by square and hexagonal elements, respectively. The generator of the q -fold symmetry, M_q , appears in the analysis of particle motions in an electromagnetic field of a certain configuration. Studies of hydrodynamic structures represent a more complicated problem; nevertheless, several of their properties may be established.

In the past decade there has been much progress towards an understanding of the transition from laminar to turbulent flow. It has become clear that there are various possible routes to this transition involving a sequence of bifurcations. The onset of turbulence in Rayleigh-Bénard convection provides a typical example³. The specific mode of break-up of laminar flows depends on the specific properties of the system⁴⁻⁷. An important common property of these mechanisms of the transition to turbulence is the existence of pre-turbulent states with coherent structures in which the chaos is either absent or weak. Alternatively, the transition to turbulence may be abrupt, the flow changing suddenly from a laminar to a strongly chaotic state with no discernible intervention of pre-turbulent states. This type of transition is observed, for example, in shear flows⁸⁻¹⁰.

It is clear that the transition to turbulence may take very diverse forms, but some features can be more or less universal in character. The first (pre-turbulent) possibility noted above is characterized by the appearance of stochastic streamlines of pre-turbulent three-dimensional structures (known as lagrangian

turbulence). Here we will be concerned with a study of this phenomenon.

We shall discuss two groups of results. First, we demonstrate analytically and numerically that steady hydrodynamic flows can have arbitrary q -fold symmetry. This establishes a correspondence between the patterns that form in fluids and those in solids. Despite the regular character of these hydrodynamic patterns, they are combined with irregular, stochastic aspects of fluid motion. Under arbitrary q -fold symmetry, streamlines are distributed chaotically in certain spatial regions. This property is associated with the second group of results presented here. Previously, only the so-called ABC (Arnold-Beltrami-Childress) flow¹¹⁻¹³, which has cubic symmetry ($q = 4$), has been discussed in terms of chaotic streamlines. The chaotic properties of the streamlines in ABC flow generate a magnetic field in a conducting fluid¹⁴⁻¹⁶, and result in spatial diffusion of a light admixture¹⁷. Here we show that three-dimensional stationary flow with q -fold quasicrystal symmetry generates a spatial web of finite thickness within which streamlines are chaotic. Our analysis implies that this situation can be considered as generic. The web separates the elements (cells) of the pattern and thereby fixes its shape. On the other hand, the web is a domain containing a stochastic distribution of streamlines and, as such, can be considered as a seed of turbulent dynamics. We obtain exact results concerning the phenomena that accompany pattern formation in continuous media, and we establish the role of (three-)dimensionality in this process.

Steady flows with quasisymmetry

The simplest non-trivial steady flows arise in two-dimensional hydrodynamics. The structures in zonal winds in the atmospheres of the Earth, Jupiter and Saturn have been related to such flows^{18,19}. Many other examples of two-dimensional self-organization are considered in ref. 20.

One may ask whether there are any general considerations in the patterns that can arise through self-organization of matter. The answer is affirmative, but only if the symmetry of the problem is restricted to certain classes. We shall demonstrate this first for the two-dimensional case, for which the Euler equation reduces to

$$\frac{\partial}{\partial t} \Delta_{\perp} \Psi + \frac{\partial(\Psi, \Delta_{\perp} \Psi)}{\partial(x, y)} = 0 \quad (1)$$

where $\Delta_{\perp} = \partial^2/\partial x^2 + \partial^2/\partial y^2$, $\Psi = \Psi(x, y)$ is the stream function and the velocity ($\mathbf{v}$) components are given by

$$v_x = -\frac{\partial \Psi}{\partial y}; \quad v_y = \frac{\partial \Psi}{\partial x} \quad (2)$$

An equation resembling (1) appears in the analysis of

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atmospheres of rotating planets¹⁸:

$$\frac{\partial}{\partial t}(\rho_s^2 \Delta_\perp h - h) - \Omega \rho_s^2 (\kappa [\mathbf{z}^0, \nabla]) h + \Omega \rho_s^4 \frac{\partial(h, \Delta_\perp h)}{\partial(x, y)} = 0 \quad (3)$$

where h is the dimensionless height perturbation of the atmosphere layer, ρ_s is the Rossby radius, $\kappa = \nabla \ln \Omega$, Ω is the Coriolis parameter and $\mathbf{z}^0$ is the unit vector along the z -axis orthogonal to the atmosphere surface. The second term in equation (3) describes a drift associated with inhomogeneity ($\kappa \neq 0$), and can be excluded by transformation to a rotating frame, thus making equations (1) and (3) equivalent. An equation similar to (3) arises in the description of drift waves in a plasma²⁰. Clearly, therefore, equation (1) describes a rather general class of physical phenomena.

Equation (1) has a steady-state solution of the form

$$\Psi = H_q = \sum_{j=1}^q \cos(\mathbf{r} \cdot \mathbf{e}_j) \quad (4)$$

where $\mathbf{r} = (x, y)$ and the vectors $\mathbf{e}_j = (\cos 2\pi j/q, \sin 2\pi j/q)$ form a q -ray regular star. The solution (4) has a number of remarkable properties. For $q=2$ we have $H_2 = 2 \cos x$ (one-dimensional Kolmogorov flow²²); for $q=4$ we obtain a flow with square cells, $H_4 = 2(\cos x + \cos y)$; for $q=3$ or $q=6$, $H_6 = 2H_3 = \cos x + \cos(x/2 + \sqrt{3}y/2) + \cos(x/2 - \sqrt{3}y/2)$ and the flow has hexagonal cells. When the vectors $\mathbf{e}_j$ do not form a regular star, equation (4) remains a steady solution to (1) but the cells can be rhombic or combinations of several rhombuses. Thus, for $q=2, 3, 4$ and 6 , equation (4) represents all known periodic flow structures with the same structure as two-dimensional crystals.

Studies of the stability of flows with crystal symmetry^{22,23} have shown that in strongly anisotropic flows ($q=2, 4$), the stability is disturbed most seriously by long-wavelength perturbations which grow when the Reynolds number exceeds a certain threshold value. Hexagonal-cell flows ($q=3, 6$) possess higher isotropy and therefore their range of stability is wider. Still more isotropic are structures of new kinds that appear for $q=5, 7, 8$ and higher. These structures are aperiodic, and have symmetries analogous to those of quasicrystals, for which structures corresponding to $q=5, 8$ and 12 have been discovered. It seems that quasicrystal-like hydrodynamic patterns could have been observed long ago, had special methods been available for their recognition. Flows with generalized quasicrystal symmetry (quasi-symmetry) constitute an intermediate phase of development, through a sequence of bifurcations, between laminar and turbulent flow.

We will demonstrate how a flow with fivefold symmetry arises. Consider, in place of (1), the Navier-Stokes equation for the stream function Ψ :

$$\frac{\partial}{\partial t} \Delta_\perp \Psi + \frac{\partial(\Psi, \Delta_\perp \Psi)}{\partial(x, y)} = \nu \Delta_\perp^2 \Psi + F \quad (5)$$

where ν is the viscosity and F is the external force that drives the motion. The steady solution of the form (4), with the quasi-symmetry obtained for the non-viscous equation (1), remains a steady solution to the viscous equation (5), provided that

$$F = -\nu \Psi \quad (6)$$

that is, when the driving force possesses the same quasi-symmetry. However, boundary conditions can have other symmetries, in which case the resulting steady flow arises from competition between these different symmetries. The surviving pattern then has either one of the imposed symmetries or some complicated combination of them.

We have solved equation (5) numerically using a 128×128 grid, and find that this suggestion is confirmed. Fig. 1 shows the results for a driving force of fivefold symmetry and boundary

conditions of twofold symmetry; that is,

$$F = F_0 \sum_{k=1}^5 \cos \left[2\pi k \left(x \cos \frac{2\pi j}{5} + y \sin \frac{2\pi j}{5} \right) \right] \quad (7)$$

$$\Psi(x, y; t=0) = \Psi_0 \cos 2\pi x$$

where $2\pi k$ is the wavenumber of the driving force and the boundary conditions are chosen to be

$$\begin{aligned} \Psi(0, y; t) &= \Psi(0, y; 0) = \Psi_0 \\ \Psi(1, y; t) &= \Psi(1, y; 0) = \Psi_0 \\ \Psi(x, 0; t) &= \Psi(x, 0; 0) = \Psi_0 \cos 2\pi x, \\ \Psi(x, 1; t) &= \Psi(x, 1; 0) = \Psi_0 \cos 2\pi x \end{aligned} \quad (8)$$

Conditions (8) impose twofold symmetry, that is, a flow that is homogeneous along the y -axis, with an x -dependent velocity gradient $v_y = \partial \Psi / \partial x$. Thus, competition arises between the twofold symmetry imposed by the boundary conditions (8) and the fivefold symmetry associated with the force (7). In Fig. 1 the parameters are chosen in such a way as to give preference to the fivefold symmetry. Intermediate cases are also possible. For the following analysis it is important to note that steady flows with quasicrystal structure (and even with pentagonal structure) can exist.

Quasi-symmetry may be typical also for three-dimensional flows described by the Euler equation

$$\frac{\partial \mathbf{v}}{\partial t} + [\nabla \times \mathbf{v}, \mathbf{v}] = -\nabla \left(P + \frac{\mathbf{v}^2}{2} \right) \quad (9)$$

where P is pressure. We consider flows that have the Beltrami property in the following simple form

$$\nabla \times \mathbf{v} = g \mathbf{v} \quad (10)$$

where g is an arbitrary scalar function of position. The vorticity fields $\nabla \times \mathbf{v}$ are steady solutions. Here we consider the case of $g = \pm 1$. If $\nabla \times \mathbf{v}$ is steady, the velocity field $\mathbf{v}$ is also steady. The components of $\mathbf{v}$ are¹³

$$\begin{aligned} v_x &= -\frac{\partial H_g}{\partial y} + \varepsilon \sin z \\ v_y &= \frac{\partial H_g}{\partial x} - \varepsilon \cos z \\ v_z &= H_g \end{aligned} \quad (11)$$

where H_g obeys $\Delta_\perp H_g + H_g = 0$, satisfying (10), and $\nabla \cdot \mathbf{v} = 0$. In particular, H_g may be defined according to equation (4). ε is a dimensional parameter associated with the z -dependent perturbation. Equations (4) and (11) indicate that the velocity field $\mathbf{v}$ has q -fold quasi-symmetry on any plane $z = \text{constant}$. Thus, quasi-symmetry is possible for three-dimensional flows. For $q=4$,

$$\begin{aligned} v_x &= 2 \sin y + \varepsilon \sin z \\ v_y &= -2 \sin x - \varepsilon \cos z \\ v_z &= 2 \cos x + 2 \cos y \end{aligned} \quad (12)$$

which corresponds to ABC flow¹¹⁻¹³ (the sign of polarization in equation (12) is taken as opposite to that in refs 11-13).

Chaos of streamlines

We now consider streamlines of steady quasi-symmetric flows. Streamlines are determined by

$$\frac{dx}{v_x} = \frac{dy}{v_y} = \frac{dz}{v_z} \quad (13)$$

where the velocity field components are given by equation (11). The streamline equations can be represented as

$$\begin{aligned} x &= x(z; x_0, y_0, z_0) \\ y &= y(z; x_0, y_0, z_0) \end{aligned} \quad (14)$$

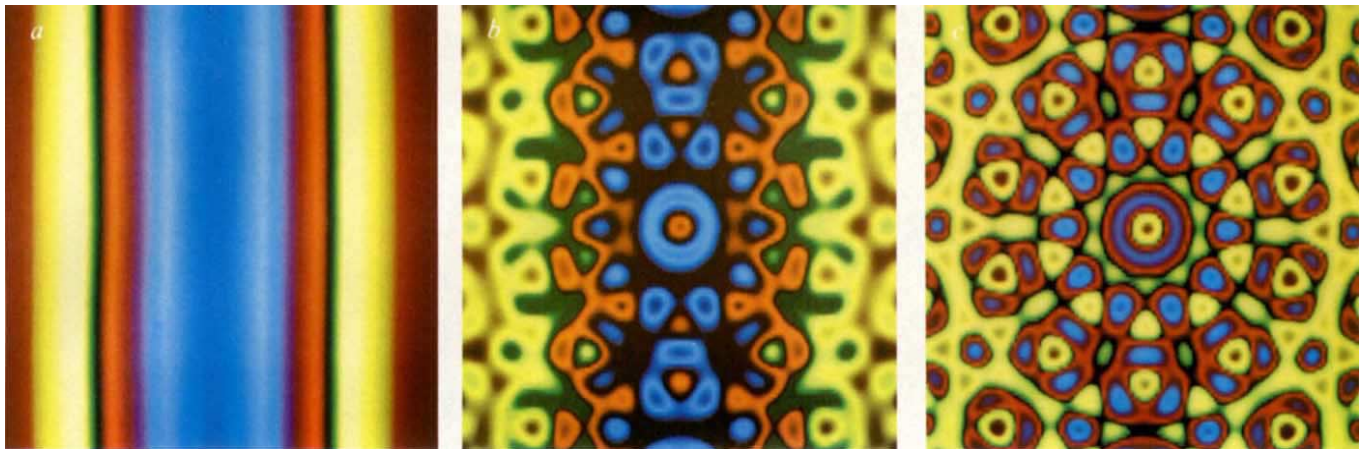


Fig. 1 Formation of a pattern with fivefold symmetry in a numerical simulation of two-dimensional flow. Boundary conditions impose twofold symmetry and induce a flow structure in the form of longitudinal strips (*a*); in time, the cells develop and the symmetry of the resulting pattern is intermediate between two- and fivefold symmetries (*b*). After some time, a steady structure appears that has fivefold symmetry (*c*). The problem solved here is given by equations (5)–(8). A computer lattice of 128×128 sites are used. The x -axis is horizontal, the y -axis is vertical, and both are shown in the interval $(0, 1)$. The colours correspond to different values of the velocity modulus. The colour scale is essentially depicted in *a*: the stream function is minimal at the centre of the middle strip and maximal at the two extremities, $x = 0$ and $x = 1$. Regions with $\Psi = 0$ are shown black. The parameters used (see equations (5)–(8)) are: $k = 10$, $F = 10^{-4}$, $\Psi_0 = 10^{-8}$ and $\nu = 0.31$. Snapshots correspond to: *a*, $t = 0$; *b*, $t = 2 \times 10^{-5}$; *c*, $t > 10^{-2}$.

Fig. 2 The stochastic web of streamlines with square symmetry ($q = 4$). Shown are the results of numerical simulations of equation (15) for $q = 4$ and $\varepsilon = 0.6$. The figure plane (x, y) corresponds to $z = \text{constant} = \pi/4$. Different streamlines (different initial conditions in equation (15)) are shown in different colours. The stochastic web, shown in yellow, is produced by a single streamline that diffuses in three-dimensional space. The web windows are occupied by invariant curves corresponding to cross-sections of stream tubes intersected by the plane $z = \text{constant}$. The size of the square is $8\pi \times 8\pi$.

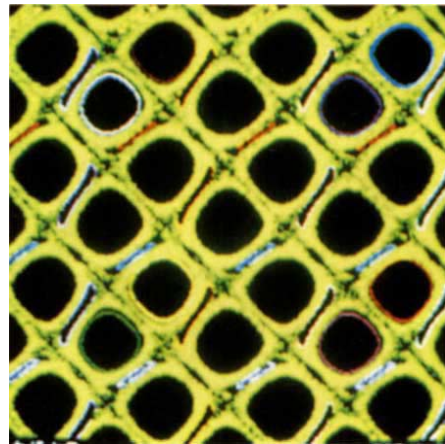
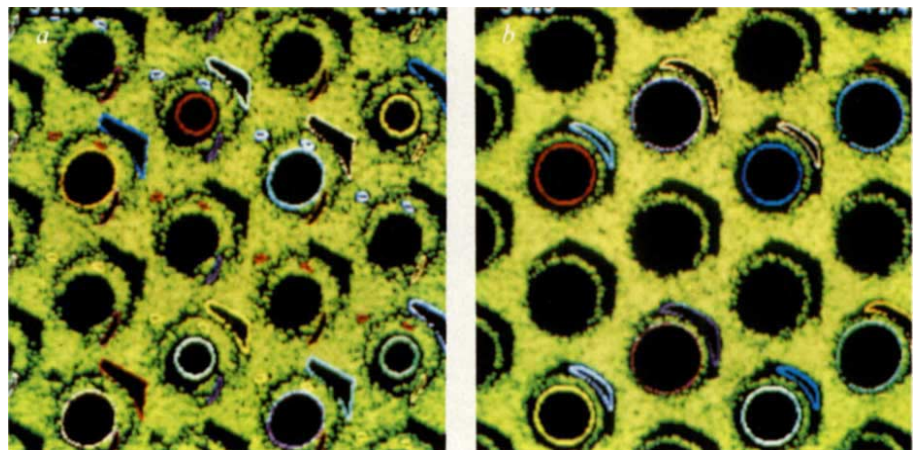


Fig. 3 Hexagonal stochastic web. This is formed in the same way as that in Fig. 2, but here $q = 3$ and $\varepsilon = 1$ (*a*); $\varepsilon = 0.3$ (*b*). The size of square is $9.2\pi \times 9.2\pi$.



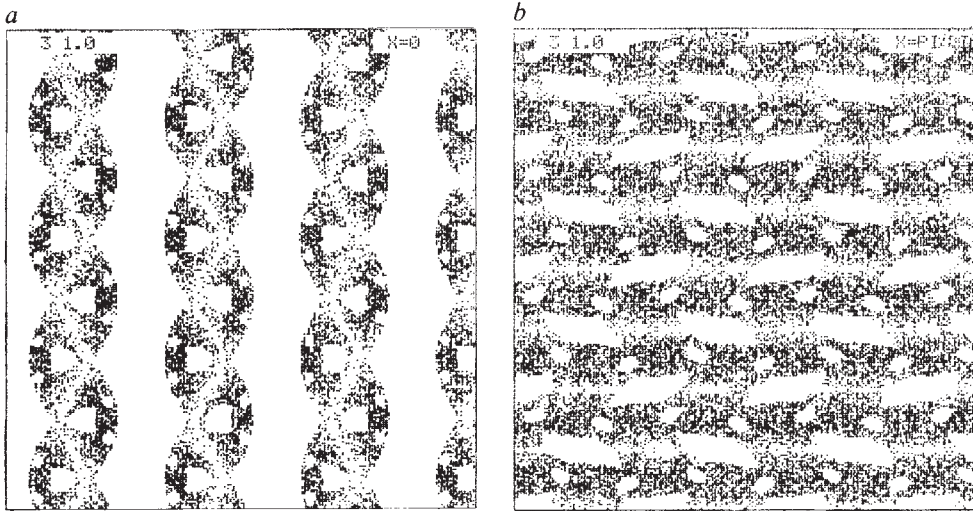


Fig. 4 Stratified structures of the hexagonal web in Fig. 3, showing the stream tubes in the (y, z) plane, for $x=0$ (a) and $x=\pi$ (b); the square size is $8\pi \times 8\pi$ in both cases.

where (x_0, y_0, z_0) is an arbitrary point on the streamline, which can be considered as an initial condition. Using equation (11), equation (13) can be written as

$$\begin{aligned} \frac{dx}{dz} &= \frac{1}{H_q} \left(-\frac{\partial H_q}{\partial y} + \varepsilon \sin z \right) \\ \frac{dy}{dz} &= \frac{1}{H_q} \left(\frac{\partial H_q}{\partial x} - \varepsilon \cos z \right) \end{aligned} \quad (15)$$

We see that the streamlines represent a dynamical system in which $\log(H_q)$ plays the role of an unperturbed hamiltonian and z corresponds to the time variable. The z -dependence of dy/dz in equation (15) is equivalent to a time-period perturbation. Moreover, (15) is equivalent to Hamilton's canonical equations. Therefore, some results of the theory of dynamical systems can be applied directly to (15). Let us briefly discuss them.

The unperturbed form of equation (15) (that is, with $\varepsilon=0$) has a manifold of singular trajectories that pass through singular points (separatrices). For $q=4$ and $q=3, 6$, all separatrices are characterized by the same value of the stream function $\Psi = H_q$ and therefore form, respectively, a unit square or hexagonal network. An arbitrarily weak periodic z -dependent perturbation (that is, arbitrarily small ε) leads to the destruction of separatrices and the appearance, in their vicinity, of a finite-thickness region characterized by stochastic streamline dynamics²⁴. This implies that a stochastic web appears in real space with square or hexagonal cells. Examples of such a web are shown in Figs 2-4, which are obtained by numerical simulation of the system of equations (15) for $q=4$ and $q=3$.

For ABC flow ($q=4$), stochastic layers were also obtained in refs 11-13. Small regions (islands) in which streamline dynamics is stable are nested within the web; that is to say, streamlines wind in a regular fashion around invariant surfaces which consist of periodically meandering tubes oriented at different angles to the plane $z=\text{constant}$. The 'windows' in the stochastic web which can be seen in Figs 2-4 correspond to cross-sections. The remainder of the region covered by the web is filled by a single trajectory. As they approach stagnation (saddle) points that are situated at the intersections of separatrices, streamlines changes direction at random; the result is spatial diffusion, similar to Brownian motion, of a particle on the cubic or hexagonal lattice. Two examples of such diffusion, for $q=4$ and $q=3$, obtained by numerical analysis, are shown in Fig. 5. A streamline can traverse a considerable distance on the plane $z=\text{constant}$, then deviate to pass over the plane $x=\text{constant}$ and trace a rather complex loop, before returning to the initial z -plane. Thus, diffusion shows 'jumps' (so-called 'Levy flight') of streamlines from one point on the plane $z=\text{constant}$ to another point on the same plane. This feature is especially clear for $q=4$ (Fig. 5a).

To estimate the thickness of the hydrodynamic web is relatively simple for weak perturbations, $\varepsilon \ll 1$, for the case $q=3$. In this case all unperturbed saddle points belong to the surface $H_3 = -1$, being solutions of the equations

$$\frac{\partial H_3}{\partial y} = \frac{\partial H_3}{\partial x} = 0$$

for which $(\partial^2 H_3 / \partial x \partial y)^2 - (\partial^2 H_3 / \partial x^2)(\partial^2 H_3 / \partial y^2) > 0$. Simple algebra shows that saddle points are given as intersections of

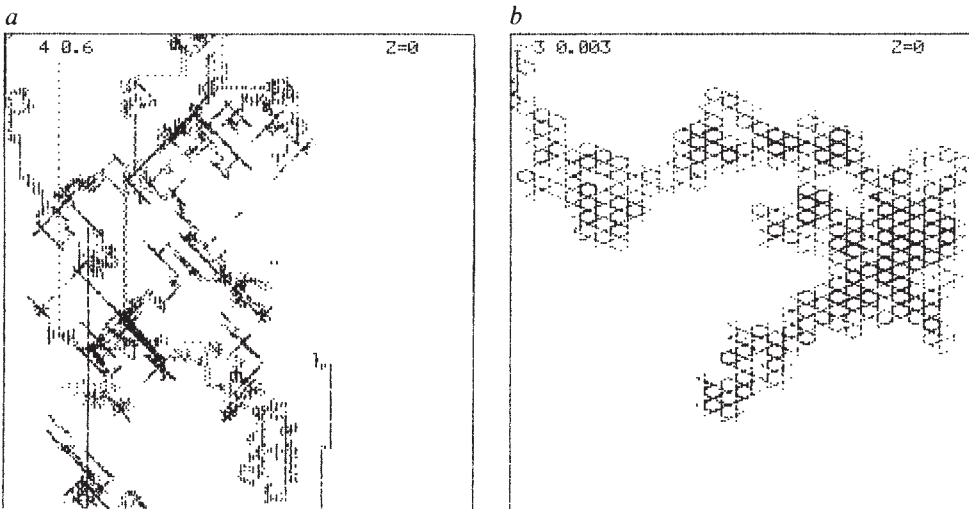


Fig. 5 Overall view of the spatial diffusion of a single streamline in the plane (x, y) , for $z=\pi/4$. a, Square symmetry ($q=4$); $\varepsilon=0.6$; $-400\pi < x < 200\pi$, $-200\pi < y < 400\pi$. b, Hexagonal symmetry ($q=3$); $\varepsilon=0.003$; $-30\pi < x < 30\pi$, $-30\pi < y < 30\pi$. The points correspond to the positions where the streamlines pass through the plane. z plays the role of a time variable, with 'time-step' 2π .

the surfaces

$$\begin{aligned}x &= \pi(2n_1 + 1); & x &= \sqrt{3}y + 2\pi(2n_2 + 1); \\x &= -\sqrt{3}y + 2\pi(2n_3 + 1)\end{aligned}$$

where $n_1, n_2, n_3 = 0, \pm 1, \dots$. These points form a hexagonal lattice on the plane $z = \text{constant}$. We now find one of the solutions for streamlines that pass through a saddle (that is, the solution corresponding to a separatrix). Consider a saddle at $x = \pi$. It follows from equation (15), with $dy/dz = \cos(\sqrt{3}y/2)$, that

$$\frac{\sqrt{3}}{2}y = \frac{\pi}{2} - 2 \tan^{-1} e^{-\sqrt{3}/2(z-z_0)} \quad (16)$$

where z_0 is the point at which $y=0$. Equation (16) can be interpreted as representing a streamline soliton which forms a hexagonal structure.

If we consider a streamline as representing the trajectory of some particle, an effective energy E can be attributed to that particle; for $\varepsilon = 0$ this energy equals H_q . For $q=3$, the variation in this unperturbed energy H_q for motion between two neighbouring saddles is

$$\Delta E \approx \varepsilon \quad (17)$$

Therefore, for small ε , the web thickness in the cross-section $z = \text{constant}$ is also of order ε .

The thickness of the stochastic web grows with ε , and for $\varepsilon \approx 1$ the regions with regular and chaotic streamlines are nearly equal in size (Fig. 3a). The region containing chaotic streamlines is fractal and, for $\varepsilon \approx 1$, it scarcely resembles a web even though it preserves the appropriate diffusive and symmetry properties.

In the case of quasi-symmetry (that is, $q \neq 2, 3, 4$ and 6), saddle points of the stream function $\Psi = H_q$ are distributed over different planes $H_q = \text{constant}$ ²⁵. Therefore, the unperturbed singular streamlines that pass through saddle points do not form a coherent, unified web even though they approach each other very closely on the plane $z = \text{constant}$, $H_q = \text{constant}$. Small, finite perturbations of order ε lead to interconnections between singular streamlines, thus forming layers with thickness of order ε . A web pattern therefore arises which has finite thickness and quasi-symmetric structure. The mechanism of formation of the quasi-symmetric web, in terms of particle trajectories, is described in ref. 2. Here, however, we encounter completely new aspects of such a system: a quasi-symmetric hydrodynamic web exists in real (x, y, z) space rather than in the particle phase space² (which includes the z -coordinate). The web is three-dimensional, with a quasi-symmetric pattern realized in the plane $z = \text{constant}$. An example of three-dimensional flow incorporating a planar quasi-symmetric pattern of fivefold symmetry is given in Fig. 6, which was obtained by numerical solution of (15) for $H_q = H_5$.

Conclusions

In summary, the examples of quasi-symmetric periodic flows considered here suggest that they represent a rather general phenomenon. The structures that arise in fluids before the onset of turbulence form a stochastic web of streamlines, the arrange-

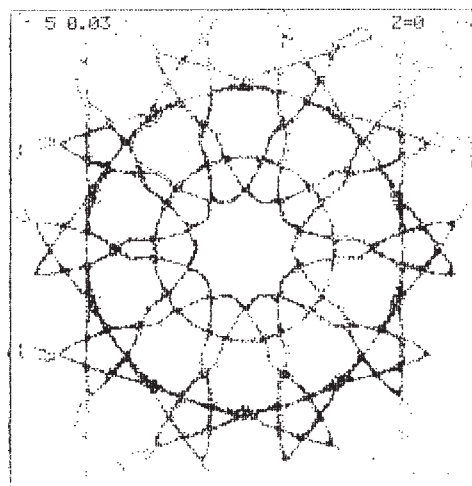


Fig. 6 Stochastic web of streamlines with fivefold quasi-symmetry, derived by solving equation (15) for $q=5$. Shown is the plane $z=0$. The points correspond to positions where the streamlines pass through the plane. The perturbation is small ($\varepsilon=0.03$), so the web channels are thin. The size of the square is $24\pi \times 24\pi$.

ment of which constitutes a distinctive pattern in real space. This general conclusion has been derived as follows. The state of a hydrodynamic system in a pre-turbulent state can be described by a finite number of harmonics in a Fourier expansion. Any finite set of harmonics possesses some kind of quasi-symmetry. In three dimensions, the streamline equations have $N=3/2$ degrees of freedom, and the stochastic nature of the streamlines must result in practically every generic situation. Provided that fluid particles follow trajectories corresponding to the streamlines, this implies that the particles have a disordered spatial distribution (that is, their motion is diffusive). This is indeed the case for steady streamlines. Particles in an admixture acquire similar properties, diffusing along the channels of the stochastic web.

In many hydrodynamic problems, weak non-stationarity of a flow cannot be excluded. We expect that in such cases the flows preserve their topological structure, because the chaotic region of the streamlines (that is, the stochastic web) has a finite range of stability with respect to weak perturbations²⁶.

Finally, we note that the expressions in (15) are very similar to the equations of motion of particles in the presence of a vortex lattice, encountered in plasma physics²⁷⁻²⁹. A vortex lattice can be formed from a system of electrostatic plane waves, with various orientations, that propagate normally to an external magnetic field. This analogy implies the important conclusion that there exist special quasi-symmetric particle trajectories in vortex-lattice systems. Particle transport occurs through the purely nonlinear mechanism of dynamical chaos rather than through seed diffusion associated with weak collisions or collective fluctuations.

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The bicoid protein is a positive regulator of *hunchback* transcription in the early *Drosophila* embryo

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A gradient in concentration of the protein product of the bicoid gene is a determinant of the anterior-posterior axis of Drosophila embryos. By binding upstream of the segmentation gene hunchback the bicoid protein controls its transcription, thereby translating maternal pattern-generating information into differential activation of zygotic gene expression.

THE molecular link between the maternal genome and the activation of zygotic gene expression is realized by maternal factors deposited in the egg during oogenesis. Three groups of maternal genes specify distinct domains along the anteroposterior axis of the *Drosophila melanogaster* embryo¹. In the anterior group the maternal gene *bicoid* (*bcd*) is crucial for the development of head and thorax². *bcd* mRNA is localized at the anterior pole of the egg and early embryo^{3,4} and the *bcd* protein comes to be distributed in a concentration gradient with a maximum at the anterior tip of the embryo⁵. The *bcd* protein gradient seems to determine position in the anterior half of the embryo in a concentration-dependent way⁶. The presence of a homeodomain⁷ in the *bcd* protein suggests that it binds to DNA in a sequence-specific way^{8,9} and thereby regulates the spatially restricted expression of zygotic target genes. The earliest zygotic genes expressed in distinct spatial domains along the anterior-posterior axis of the embryo are the gap genes¹⁰. The phenotype of mutations in the gap gene *hunchback* (*hb*; deletion of gnathal and thoracic segments¹¹), and the expression pattern of *hb* RNA in *bcd* mutant embryos¹² identify *hb* as a probable target for *bcd* regulation. Here, we show that the *bcd* protein binds to five sites upstream of the transcription start site of the zygotic gap gene *hunchback*. These binding sites define the consensus binding sequence: 5'TCTAATCCC3'. Transient expression assays in embryos as well as in tissue culture cells reveal that the three binding sites in the promoter region (−50 to −300) are necessary and sufficient for the activation of zygotic *hunchback* expression.

Mapping *bcd* protein binding sites

In the syncytial blastoderm *hb* expression occurs in two domains (ref. 13, see also Fig. 3a). The 2.9-kilobase (kb) transcript in the anterior domain is under the control of a separate promoter and is not expressed in *bcd* mutant embryos¹². The regulatory region necessary for correct spatial expression of the zygotic 2.9 kb *hb* transcript has been mapped using p-transformation of *hb* β -galactosidase gene fusions¹⁴ to a 700-base pair (bp) DNA fragment that includes the first intron and 300 bp upstream of the transcription start site.

Full-length *bcd* protein was expressed in *Escherichia coli* by generating a *Nde*I site at the start ATG of the longest open reading frame and cloning *bcd*-protein-coding sequences into the pAR3038 expression vector¹⁵. The same cDNA was also expressed in *Drosophila* Schneider cells using the actin 5c promoter (Fig. 1a). The protein derived from *E. coli* migrates slightly faster (lane 3, apparent relative molecular mass M_r = 53,000 (53K)) than that from Schneider cells (lane 2, 58K) or embryos (lane 1, 56–60K). Different electrophoretic mobilities can be explained by post-translational modification. To test

whether the decrease in electrophoretic mobility is due to phosphorylation (see for example *engrailed* (*en*)¹⁶, *Ultrabithorax* (*Ubx*), L. Gavis, personal communication), we immunoprecipitated *bcd* protein transiently expressed in Schneider cells which had been grown on [³²P] orthophosphate-containing media. The immunoprecipitate was analysed by SDS-PAGE. A ³²P-labelled protein migrated at the expected size of the *bcd* protein (Fig. 1b, lane 2). Treatment of immunoprecipitates from Schneider cells transiently expressing *bcd* protein with potato acid phosphatase results in a decrease in apparent M_r of the immunoprecipitated protein (lane 4, 5), which now co-migrates with the *bcd* protein expressed in *E. coli* (lane 3). In addition, multiple bands of intermediate apparent M_r are also visible. We conclude that the *bcd* protein in *Drosophila* Schneider cells, and probably also in the embryo, is subject to multiple phosphorylations.

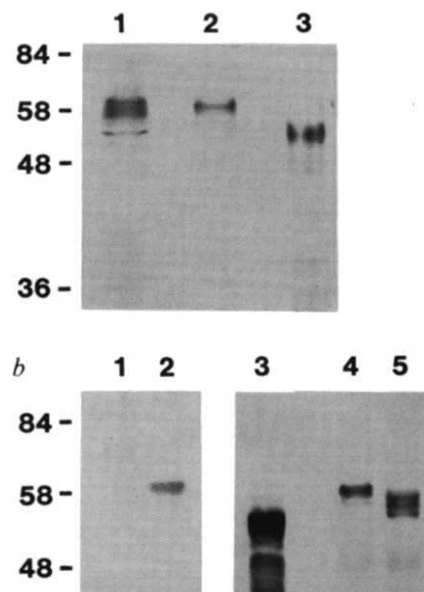
We used an immunoprecipitation assay¹⁷ to screen large regions of genomic *hb* DNA for *bcd*-binding sites (see Fig. 2). *E. coli*-derived full-length *bcd* protein was immunoprecipitated with polyclonal antibodies bound to fixed *Staphylococcus aureus* cells and the resulting immunoprecipitate was incubated with end-labelled restriction fragments from the *hb* promoter. Two restriction fragments from the *hb* gene were bound with high affinity: fragment A (−298 to −55) and B (−2,172 to −2,631) base pairs upstream from the transcription start site of the zygotic 2.9 kb *hb* transcript (Fig. 3a). A third fragment, C, from −4,242 to −3,890 base pairs upstream from the 2.9 kb transcription start site (or −1,020 to −672 upstream from the 3.2 kb transcript) was bound with relatively low affinity. Fragment A binds with the highest affinity as, even at the highest concentrations used, salmon sperm DNA had a negligible effect on the amount of fragment A bound. At this competitor concentration the binding of fragment B is reduced to about one-third and binding of fragment C is eliminated completely. The same fragments were also precipitated using *bcd* protein expressed in Schneider cells (data not shown), indicating that phosphorylation does not affect binding specificity in this assay.

Consensus sequence for *bcd* binding

The individual binding sites were analysed further by DNaseI footprint experiments¹⁸. The results are shown in Fig. 4 and summarized in Fig. 3. Fragment A contains three regions which are protected against DNaseI digestion by *bcd* protein, A1 (−280 bp), A2 (−170 bp) and A3 (−65 bp). A2 consists of two protected regions, one well protected (around −170) and one weakly protected (around −187). The A region is part of the 700 bp fragment shown to be sufficient for the correct spatial expression of the 2.9 kb *hb* transcript¹⁴. The B fragment consists of two binding regions, B1 (about −2,325) and B2 (about

Fig. 1 *a*, Expression of bcd full-length protein in *E. coli* and *Drosophila* Schneider cell tissue culture. Western blot of extracts separated by PAGE, probed with anti-bcd antibodies. Lane 1, extracts from 2- to 3-hour-old *Drosophila melanogaster* embryos (500 µg protein); lane 2, extracts from the Schneider cell line S2/M3 that transiently expresses bcd under the control of the *Drosophila* actin 5c promoter (plasmid pAcbcdEE, 100 µg protein applied); lane 3, extracts from *E. coli* that express bcd under the control of the T7 phi 10 promoter (plasmid pARbcdNB, 10 µg protein applied); M_r s are indicated on the left, in thousands. *b*, Modification of bcd protein by phosphorylation. The bcd protein was transiently expressed in Schneider cells grown in the presence of [32 P]orthophosphate. Immunoprecipitation from nuclear extracts with anti-bcd antibody and autoradiography of the precipitate resolved on SDS-PAGE demonstrates that in bcd-expressing cells (lane 2) but not in non-expressing cells (lane 1) a phosphorylated form of bcd protein is synthesized. Incubating bcd immunoprecipitates from extracts of Schneider cells transiently expressing bcd protein with potato acid phosphatase results in an increase of the electrophoretic mobility of bcd protein. The most rapidly migrating form is of the same size as the bcd protein expressed in *E. coli* (lane 3) as revealed from western blot analysis of the reaction products: bcd protein incubated without (lane 4), and with (lane 5) potato acid phosphatase.

Methods. The *bicoid* gene gives rise to a primary transcript that is subject to differential splicing. The alternatively spliced RNAs code for different protein products^{3,5}. In this study we used only one protein species derived from cDNA c53.46.6 (ref. 3). Although it is not yet clear whether the different forms of bcd protein are functionally equivalent, RNA injections into early embryos reveal that this bcd protein species can rescue all aspects of the mutant *bicoid* phenotype (our unpublished data). For the protein expression constructs, *bcd* cDNA c53.46.6c was used (ref. 3; all the base pairs that differ from the genomic sequence and give rise to amino-acid substitutions were exchanged with genomic sequences: detailed protocol on request). Construction of pARbcdNB: the *EcoRI*-*Sall* fragment from c53.46.6c was used to generate a *NdeI* site at the initiator ATG of the longest open reading frame by oligonucleotide site-directed mutagenesis (using the pMa/c 254 plasmid vectors, Stanssens and co-workers, unpublished results), the cDNA was reconstructed and a *Bam*HI site generated at the *Xma*I site 3' to the open reading frame; the *NdeI*-*Bam*HI fragment was cloned into the expression vector pAR3038 (ref. 15). Construction of pAcbcdEE: the *EcoRI* fragment of cDNA c53.46.6c was blunt-end-ligated into the *Bam*HI site of the vector pPAC downstream of the actin 5c promoter and upstream of the actin 5c polyadenylation processing signals (vector pPAC constructed by H. Lipshitz using the *Drosophila melanogaster* actin 5c gene, ref. 28). bcd protein was expressed from pARbcdNB (ref. 15) in *E. coli* BL 21 DE3. *Drosophila* tissue culture and transfection techniques were as described²⁹. Phosphate labelling was performed using 0.5 mCi 32 P-labelled NaH_2PO_4 per 5 ml of medium in a 6 cm petri dish. bcd protein was immunoprecipitated from cleared lysates of isolated nuclei sonicated in 20 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.5% NP-40, 2 mM phenylmethylsulphonylfluoride using polyclonal anti-bcd antibody prebound to *Staph. aureus* (Calbiochem). The immunoprecipitates were treated with 50 µg ml⁻¹ DNaseI (Cooper), 10 mM MgCl₂ in the above buffer for 15 min on ice. Potato acid phosphatase treatment (20 µg per 100 µl) was as described³⁰. Extract preparation from *Drosophila* embryos, electrophoresis and further processing were as previously described⁵.



-2,280). Both are less well protected than the A sites. No footprint could be obtained with the C fragment (data not shown), but DNaseI hypersensitive sites could be identified around -4,100 and -3,998. Analysis of the sequences of fragment A and B revealed that each of the sites contains a sequence approximating a 9-bp consensus sequence TCTAATCCC (Fig. 3b). This sequence is best conserved in sites A2 and A3 whereas A1, B1 and B2 each contain mismatches with the consensus. Only the central part of the sequence, TAATC is conserved in all five sites. Using binding conditions of low stringency (no competitor DNA) hypersensitive sites could be detected around CTAAT or TAATC sequences (for example -4,100, -3,998) or even at TAAT alone (data not shown). In addition, within fragment A in the region -241 to -204 on the sense strand some hypersensitive sites do appear and one can detect weak protection of some bases on the antisense strand. Three sequence motifs in this region (TGCTAAGCT, -241 to -233; CGCTAAGCT, -225 to -217; and GATCATCC, -212 to -205) resemble the consensus sequence (underlined nucleotides) weakly. Notably, all three motifs are palindromic. The bcd protein seems to bind to DNA sequences that contain elements like TAAT, CTAA, CTAAT, TAATC, where the context of the surrounding nucleotides determines the affinity of binding, the best fit so far identified being the consensus TCTAATCCC.

For a few other *Drosophila* homoeodomain-containing proteins, sequence-specific binding has been demonstrated and the binding sites seem to lack palindromic character as does the bcd consensus sequence. Engrailed (*en*), fushi tarazu (*ftz*), zen (*zen*) and even skipped (*eve*) protein *in vitro* all bind *en* upstream sequences with the consensus TCAATTAAT^{20,21}. In addition *eve* protein recognizes TCAGCACCG sequences in the *eve* upstream region²¹. Ubx protein binds to (TAA)_n repeats (P. Beachy, M. Krasnow, L. Gavis and D. Hogness, personal

communications). Analysing the 'contact amino acids' of the homoeodomain that are probably exposed to DNA (positions 1, 2, 5, 6 and 9 within the recognition helix of the helix-turn-helix motif²², one finds that Ubx, ftz, zen and en are identical in all but the second amino acid, providing an explanation for their similar binding characteristics. In contrast, bcd differs in positions 1, 2 and 9 with respect to the above proteins. Further analysis will show if bcd has overlapping binding characteristics with other *Drosophila* homoeodomain proteins.

Embryonic expression assay

To investigate the influence of the bcd-binding sites on *hb* transcription, we used an embryonic transient expression assay (Fig. 5). The *hb* promoter fragments, including increasing numbers of bcd-binding sites, were fused to the chloramphenicol acetyltransferase (CAT) gene at position +107 of the 2.9 kb *hb* transcript. The plasmid constructs were injected into the anterior half of early cleavage embryos and the expression of the reporter gene CAT was measured at the onset of gastrulation. We expect that a small amount of the plasmid DNA ends up in a nuclear location during the rapid early cleavage cycles and comes to be expressed under conditions similar to those that lead to the expression of the chromosomal *hb* copies.

On injection of the *hb*-CAT constructs into early embryos, we do indeed observe a bcd-dependent expression of CAT. Figure 5 shows that with the longest upstream sequences CAT expression is about 50 times higher in embryos from wild-type females than in embryos derived from *bcd*⁻ females. Elimination of the binding sites B1 and B2 did not significantly affect the level of expression. In contrast the successive elimination of the A sites results in a dramatic reduction of the bcd-dependent *hb*-CAT expression. If all binding sites (but not the TATA box) are removed, no significant expression is observed.

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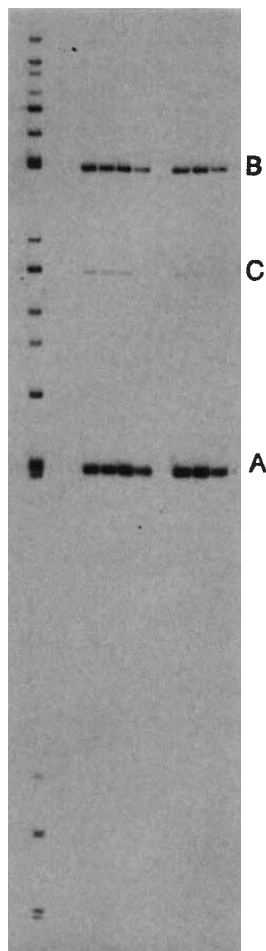


Fig. 2 Specific binding of bcd protein to restriction fragments from the *hb* gene. The genomic 7.5 kb *Bam*HI–*Eco*RI fragment contains the zygotic 2.9 kb transcript that is expressed in the anterior gap domain and about 4.5 kb of upstream sequences¹². It was digested with a set of restriction enzymes to produce fragments of 100–1,000 bp and the fragments were end-labelled (lane 1). bcd protein expressed in *E. coli* under the control of T7 RNA polymerase (see Fig. 1), was used to immunoprecipitate fragments that bind specifically to the protein. Lane 2 shows a control reaction using anti-bcd immunoprecipitates from non-bcd expressing bacteria; no fragments were bound. In the immunoprecipitates with bcd protein (lanes 3–9), two fragments (A, a 243-bp *Sal*I–*Mlu*I fragment from –298 to –55 with respect to the transcription start site; B, a 459-bp *Hind*III–*Bgl*II fragment from –2,172 to –2,631) were bound with high affinity, one with relatively low affinity (C) a *Bam*HI–*Hind*III fragment from –4,242 to –3,890). To assess the specificity of binding, zero (lane 3), 0.25 (lane 4), 1 (lane 5) and 5 (lane 6) μ g of competitor DNA (salmon sperm DNA, 1,000-fold excess over total labelled DNA, 40,000-fold excess over one specific labelled fragment, lane 6) were included in the binding reaction. Binding of fragments A and B is only slightly affected at the highest competitor concentration, fragment C is no longer bound under this condition. In the experiments shown in lanes 7, 8 and 9, after 45 min binding the immunoprecipitates were washed and competitor DNA was added for 10 min (0.2, 1 and 5 μ g, respectively). The off rates for binding to fragment A and B are apparently rather low.

Methods. The genomic 7.5 kb *Bam*HI–*Eco*RI fragment was isolated from the plasmid pE8-B1000A (obtained from D. Tautz, ref. 13), digested with a set of restriction enzymes and end-labelled with polynucleotide kinase (restriction enzymes: *Ava*II, *Bgl*II, *Mlu*I, *Hind*III, *Nde*I, *Sal*I and *Nco*I; no additional fragments were precipitated when the same experiments were performed using either a *Hinf*I or a *Taq*I digest). bcd protein was expressed in the bacterial strain BL21(DE3) as described in ref. 15. Induced cells were collected by centrifugation and resuspended in 1:200 volume of buffer Z (ref. 21), incubated for 15 min on ice and sonicated twice for 15 s. The lysate was spun clear and the supernatant taken as extract. Immunoprecipitation was carried out according to a modified procedure⁹. Fixed *Staph. aureus* cells (Calbiochem, 100 μ l of 10% suspension) were washed twice with binding buffer BB (20 mM Tris pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, 10% glycerol) and incubated with polyclonal anti-bcd antibody (20 μ g in 500 μ l BB) for one hour on ice. The suspension was washed twice with BB. Extracts (100 μ l) from bacteria carrying pARbcdNB or pAR3038, respectively, were added in 1 ml BB, 2 mM PMSF and incubated for two hours on ice. The immunocomplexes were washed twice with BB, once with BB 0.2% NP-40 and resuspended in 100 μ l BB. Binding reactions were in 50 μ l total volume including 10 μ l immunocomplex suspension, NaCl added to 170 mM, 5 ng end-labelled fragments and various amounts of sonicated salmon sperm DNA as competitor for 40 min on ice. For lanes 7–9, immunoprecipitates were washed once with BB and incubated for 10 min in the above reaction mixture with the indicated amounts of competitor DNA. Precipitates were washed four times with 1 ml BB 0.2% NP-40 each, resuspended in 100 μ l 10 mM Tris pH 7.5, 1 mM EDTA, 5 μ g sonicated salmon sperm DNA was added as carrier, the fragments phenol/chloroform extracted, precipitated and separated on a 4% denaturing polyacrylamide gel. Autoradiography was performed using Cronex 4 films.

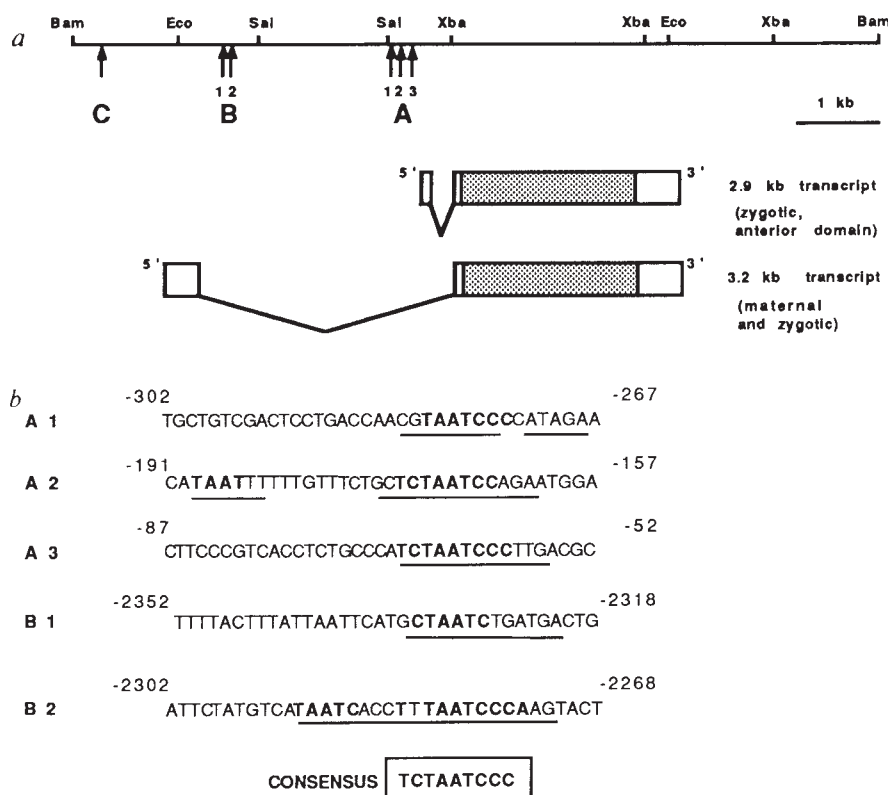


Fig. 3 a, Map of the *hb* gene indicating the locations of bcd-binding sites. During the syncytial blastoderm stage *hb* expression occurs in two domains which are under separate regulation. The 2.9 kb *hb* transcript is expressed in an anterior domain which extends from 55–100% egg length, whereas the 3.2 kb transcript (which is expressed maternally and zygotically) is localized to 0–25% egg length¹³. In addition, the 3.2 kb transcript can be detected in a narrow stripe at about 53% egg length shortly before the onset of cellularization. A, B and C are the fragments identified in the experiment shown in Fig. 2. b, Nucleotide sequences of the regions where bcd protein binds to *hb* regulatory regions. Base pairs protected against DNase I digestion as referred from Fig. 4 are indicated by a bar below the sequence. Comparison of the protected sequence elements led to the identification of a consensus binding sequence. Bases in the protected regions that fit the consensus sequence are in bold.

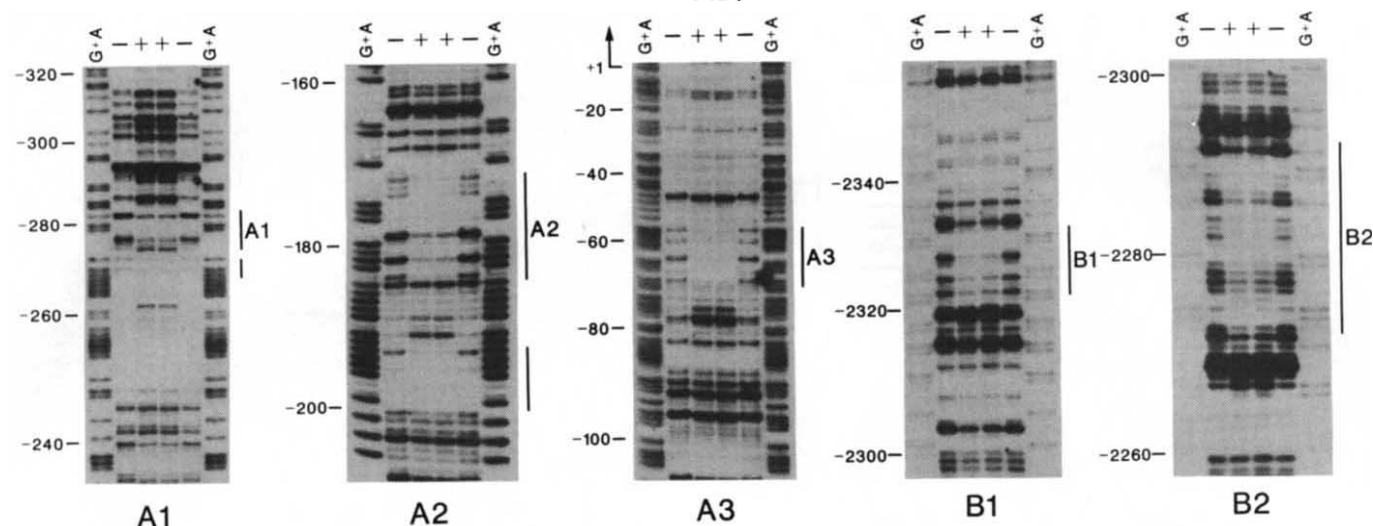


Fig. 4 Analysis of *bcd* protein binding to *hb* sequences by DNaseI footprinting. The DNA fragments identified in the immunoprecipitation assay, the sequences surrounding these fragments as well as the intron of the 2.9-kb transcript were analysed for *bcd* protein binding sites by DNaseI footprinting. Specific binding could only be detected in the same regions that were identified by the immunoprecipitation assay, regions A and B. Region A seemed to consist of three *bcd* binding sites, A1, A2 and A3, each spaced at about 100-bp intervals. Region B consists of two binding sites, about 40 bp distant from one another. Lanes show (G+A) sequence marker and footprint reactions with (+) and without (-) *bcd* protein. Positions are indicated on the left side of each panel in base pairs distance from the transcription start site. Protected regions are marked by a bar on the right side.

Methods. For DNaseI protection assays (ref. 31) *bcd* protein was affinity purified as described in Fig. 2 and resuspended in 100 μ l FPBB²¹ (110 mM KCl, 47 mM HEPES pH 7.8, 12 mM MgCl₂, 0.05 mM EDTA, 1 mM dithiothreitol, 17% glycerol and 0.02% NP 40). Genomic fragments were asymmetrically labelled with Klenow and radioactive dNTPs (A1: labelled at *Mlu*I site -55, linearized at *Nsi*I site -434, sense strand; A2 and A3: labelled at an *Eco*RI site generated at *Sal*I-298 and linearized at *Xba*I+431, antisense strand; B1 and B2 labelled at an *Eco*RI site generated at the *Bgl*III site -2,170 linearized at *Hind*III -2,631, sense strand). Binding reactions were done with 4-8 ng of labelled fragment and 20 μ l of immunocomplex in a total volume of 50 μ l FPBB for 45 min on ice. 50 μ l 10 mM MgCl₂/5 mM CaCl₂ were added to the reaction followed by 5 μ l of freshly diluted DNaseI (Worthington) at a final concentration of 5 μ g per ml reaction mix. After 5 min on ice, digestion was stopped by the addition of 110 μ l 1% SDS, 20 mM EDTA, 200 mM KCl, 250 μ g per ml yeast tRNA (65°C). After phenol/chloroform extraction and ethanol precipitation samples were electrophoresed on 8% polyacrylamide/7.5 M urea gels and visualized by autoradiography.

We also expressed the CAT constructs in Schneider cells. In this assay, there is a substantial expression of CAT even in the absence of *bcd*. Co-transfection (M. Krasnow and D. Hogness, unpublished results) with the *bcd* transcription unit under the control of the actin 5c promoter results in a fivefold stimulation of CAT expression using constructs that contain the whole A region (Fig. 5d). The strong reduction in expression when removing only the A1 site supports the idea that the three sites cooperatively activate *hb* expression. Deletion of A1 and A2 decreases the expression to background levels.

Discussion

The concerted action of three regulatory sites, each 100 bp apart, raises the question of how they interact with the distant transcription initiation complex. In prokaryotes, loop formation between promoter elements has been visualized on a molecular level²³. In eukaryotes, the chromatin structure might influence the spatial arrangement of regulatory regions. The analysis of the *Drosophila* 26K heat-shock protein promoter²⁴ demonstrates that the positioning of a nucleosome might bring regulatory sequences together on its surface²⁵. The spacing of *bcd*-binding sites A1-A3 might allow a similar mechanism. Remarkably, in *Drosophila virilis*, not only the *bcd*-binding sites, but also their spacing seem to be conserved (D. Tautz, personal communication). A close spatial proximity of the binding sites might be the basis for cooperativity in activation of transcription.

An interesting question is whether *bcd* protein provides only the DNA-binding function or also the transcription-activating functions that interact with the cellular transcription machinery. In yeast, transcriptional activators are characterized by acidic protein domains²⁶ and these acidic regions seem to function in

other eukaryotic systems as well²⁷. A good candidate for a transcriptional activator domain within the *bcd* protein is the region between amino acids 345 and 390 (see ref. 3 for protein sequence) that has 10 negative charges and in addition several putative phosphorylation sites. As noted above, the protein is phosphorylated in *Drosophila* Schneider cells. The level of phosphorylation might influence the strength of the transcriptional activator function of *bcd*.

The molecular interaction between the *bcd* protein and the *hb* promoter is probably one of the first events in gene regulation during embryogenesis and shows how a maternally derived regulatory protein, *bcd*, participates in initiating the process of pattern formation. A major question about the interactions between maternal coordinate genes and zygotic segmentation genes is how the information provided by the smoothly graded distribution of a maternal gene product like *bcd* is transformed into the more sharply defined gap gene domains. In the wild-type, the early posterior border of *hb* expression is at 55% egg length¹². In this region of the embryo the *bcd* gradient is already very shallow, although the *bcd* protein is detectable up to 30% egg length⁵. Several models might explain how the transition from a shallow gradient to a sharp on/off decision is achieved. For example, other so far unidentified factors might compete for *bcd*-binding sites or inhibit *bcd* binding and thereby sharpen the posterior border of *hb* expression. So far there is no genetic evidence for the existence of genes that could function as competitors^{1,2,6}. Our data support models that include cooperativity as an important mechanism to control the spatially restricted expression of zygotic target genes. A precedent for the role of cooperativity in gene regulation is the action of λ repressor (for review see ref. 19). The three *bcd*-binding sites identified in the

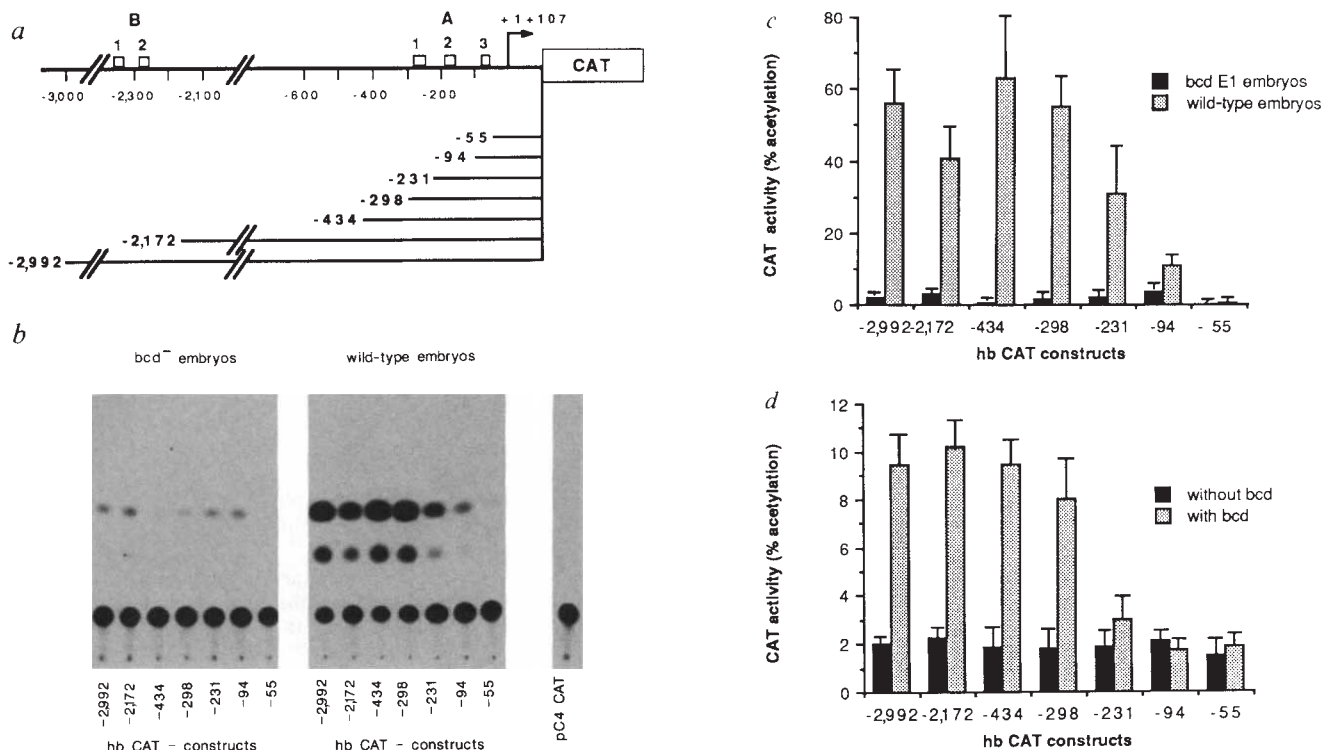


Fig. 5 Influence of *hb* upstream sequences on the expression of a reporter gene coding for CAT as assayed by transient expression in embryos and Schneider cell tissue culture. **a**, Map of *hb* CAT fusion genes used in this analysis. *hb* upstream sequences and the *hb* transcription start site plus 107 bp of the non-translated leader were fused to the CAT gene. Construct *hb*CAT-55 ends just downstream of the A3 binding site, *hb*CAT-94 includes the A3 binding site, *hb*CAT-231 includes A2 and A3, *hb*CAT-298 the whole A cluster. To analyse a possible function of the B cluster, we made constructs ending upstream and downstream of that cluster. **b**, Embryonic transient expression assay. *hb*CAT fusion gene plasmids were injected into early cleavage embryos and the embryos analysed for expression of CAT at the onset of gastrulation. The left part shows CAT assays using embryos from homozygous *bcd*^{E1} females (control with no *bcd* protein) and the right part those from wild-type embryos. pC4 CAT marks the control when just injecting the vector alone without any *hb* sequences. **c**, Quantitative analysis of the embryonic transient expression assay. Percentage of acetylated forms of total chloramphenicol were plotted for the expression of CAT in wild-type and *bcd*^{E1} embryos. The stimulation of expression in wild-type embryos compared to *bcd* mutant embryos is about 50-fold when all three sites of the A cluster are present, 10-fold with just A2 and A3 and 3-fold when only A3 is present. Comparing the construct that has no *bcd*-binding site (*hb*CAT-55) with the one that contains all three A sites (*hb*CAT-298) gives a 200-fold stimulation of expression. The presence or absence of the B cluster does not seem to have a significant influence on the expression starting from the zygotic 2.9 kb *hb* transcript start site. **d**, Transient expression of *hb*-CAT fusion genes in *Drosophila* Schneider cells co-transfected with *bcd* under the control of the actin 5c promoter (pPacbcdEE, see Fig. 1). Correction for variation in transfection efficiency was achieved by co-transfection of an actin 5c *LacZ* fusion gene and assay of β -galactosidase activity in the cell extracts. The graph shows activation of CAT expression when comparing transfections with or without *bcd*. All *hb*-CAT constructs showed a high background expression in the absence of *bcd*. The presence of *bcd* protein leads to a fivefold stimulation of CAT expression from constructs containing all three A sites. Removal of A1 results in a strong reduction of expression. CAT expression in the presence of just A3 is no more different from background levels. Northern analysis of RNA from cells transfected with similar *hb lacZ* fusion genes indicate an about fourfold increase in transcript level comparing the *hb*-298lacZ to the *hb*-55lacZ construct.

Methods. A *Bam*HI site was generated at the *Taq*I site within the zygotic 2.9-kb *hb* transcript (+107) by ligating a *Bam*HI linker to the filled in *Taq*I site. Genomic *hb* DNA was digested with enzymes cutting at the sites indicated in **a**, blunt-ended and then cut at the newly introduced *Bam*HI site (+107). The CAT reporter gene vector pC4 CAT (ref. 32) was linearized at the *Sal*I site in the polylinker, blunt-ended and cut with *Bam*HI. Isolated *hb* promoter fragments were cloned into the pC4 CAT vector by standard techniques. Each plasmid (5 pmol) was dissolved in 0.1 ml sterile water containing 10^6 c.p.m. 32 P dCTP. Injections into early cleavage embryos, 100 of each mounted on one coverslip and covered with Voltalef 10S oil, were according to standard procedures². The embryos were left to develop at 18 °C until the onset of gastrulation, washed off the coverslip with heptane, washed carefully three times with heptane to remove last traces of oil and the heptane was evaporated completely with an air stream. Radioactivity injected into the embryos was determined and the embryos were frozen in liquid nitrogen. An average of 70 nl was injected into each 100-embryo batch (about 7% of the egg volume). Batches with injected amounts more than 30% different from the average were discarded. For each wild-type construct four groups of 100 embryos and for the *bcd*^{E1} control as well as the vector control two groups of 100 embryos were analysed. Modified CAT assays were used³³. 100 μ l of 0.25 M Tris pH 7.8 was added to each batch, the embryos sonified for 5 s, incubated at 65 °C for 5 min and insoluble material discarded after centrifugation. Aliquots (25 μ l) of the extract were mixed with 54 μ l 0.25 M Tris pH 7.8, 20 μ l 4 mM acetylCoA and 1 μ l [14 C]chloramphenicol (0.2 μ Ci). The reaction mixture was incubated for one hour at 37 °C, the chloramphenicol extracted with ethyl acetate (0.5 ml), the ethyl acetate evaporated and the acetylated forms of chloramphenicol separated by TLC. The acetylated and non-acetylated forms of chloramphenicol were visualized by autoradiography and quantitated by scintillation counting. Bars in **c** show mean values of the determinations and the standard error of the mean. *Drosophila* tissue culture and transfection techniques were used²⁹. The pPacLacZ plasmid was constructed by inserting the blunt ended *Sal*I-HindIII fragment from pCaSpeR AUG β GAL (ref. 32) containing the coding region for β -galactosidase and the SV40 polyadenylation site into a blunt-ended *Sal*I-digested pPac actin 5c promoter vector (H. Lipshitz, unpublished results). Co-transfections were performed using 5 μ g pPacLacZ, equimolar amounts of the *hb*-CAT constructs (1–1.5 μ g) with or without 10 μ g of pPacbcd. pUC 18 DNA was added to reach 20 μ g total DNA per transfection. Cells from one 6-cm plate were washed in PBS and lysed in 200 μ l 250 mM Tris pH 7.8 by sonication. Cleared lysates were assayed for β -galactosidase activity using o-nitrophenyl- β -D galactopyranoside as substrate. The measured activities were used to correct the amount of extract used in the CAT assay (see above) for the variation in transfection efficiency. The graph shows mean values of two determinations and the mean errors.

hb promoter might allow cooperative binding and thereby full activation of transcription above a low threshold of bcd concentration. Indeed, the level of *hb* expression is constant within the anterior 40% of the embryo¹², independently of the marked increase in bcd protein concentration towards more anterior positions. In addition, a cooperative mechanism can generate an on/off transition within a relatively small range of bcd protein concentrations. One would not expect that such a mechanism immediately generates a sharp border; indeed, initially the posterior border of *hb* expression is not clearly defined within a stripe two or three nuclei wide¹². Subsequent regulatory interactions between *hb* and other zygotic genes might be involved in the establishment and maintenance of a border as sharp as that observed in later blastoderm stages¹².

Genetic analysis suggests that bcd regulates more than one zygotic target gene in a spatially restricted way^{2,6}. Different

spatial limits of target gene expression could be achieved if the affinities of their bcd-binding sites vary. Thus a reduced affinity for bcd-protein binding would restrict target gene expression to more anterior regions which contain higher levels of bcd protein. Analysis of further target genes will show how bcd protein fulfils its function as a morphogen at the molecular level.

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LETTERS TO NATURE

Strong variability of weak-core radio sources

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The variability of radio sources whose emission is dominated by single unresolved cores (core-dominated sources) is well established¹. Evidence is also mounting that sources such as Cen A and 3C111, which are not core-dominated, also have varying cores²⁻⁴. Statistical studies of source fluxes using single-dish data and comparisons with known morphologies suggest that even in sources whose emission is swamped by that of the extended emission, the cores can be highly variable on timescales of weeks to years^{5,6}. Here we present direct observational evidence for core variability of two lobe-dominated (weak-core) radio sources, GT1945+241 and GT0304+575. In the context of the beaming model, weak-core sources have little Doppler boosting and any residual variability is mainly intrinsic. The extreme nature of the core variability of the two sources described here suggests that the intrinsic variability can dominate beaming-related variability. If future studies reveal that this type of source is common, aspects of the beaming model, particularly in the context of the Unified Scheme, would be brought into question.

Confirmation of weak-core variability through direct measurements of the core fluxes bears directly on the relationship between the core-dominated sources and the weak-core sources. The advent of the beaming model^{7,8} has led to the 'Unified

Scheme'⁹, in which the two classes of sources differ only because of geometry. Sources with major axes close to the line of sight towards us. The Doppler boosting associated with relativistic beaming amplifies the synchrotron radio emission. The beaming itself increases the degree of variability and, through time dilation effects, reduces the variability timescales³. Sources with major axes well away from the line of sight should show minimal beaming effects. These sources should therefore have fainter cores, fewer of them should be variable and the timescales of variability should be, on average, greater. A competing view holds that the two classes of sources are intrinsically different, as suggested by their differing spectra and polarization properties¹⁰. Unambiguous detection of strong, short-term variability in weak-core sources would argue against a straightforward interpretation of the Unified Scheme and in favour of the two-class scenario.

Here we present direct evidence for such variability in two specific sources, GT1945+241 and GT0304+575. These sources are taken from a sample of variable radio sources from the 6-cm galactic-plane survey of Gregory and Taylor¹¹. The sources were selected solely on the basis of their variability, with no prior knowledge of their morphologies. Because the sources are all in the galactic plane it has not been possible to identify them optically. Distances are therefore not known and the possibility exists that the one or both of the sources discussed here are galactic. Statistical studies of source counts suggest, however, that ~90% of the survey sources are expected to be extragalactic¹³. Furthermore, recent H I absorption measurements (made by us) indicate degrees of absorption by foreground gas that are large enough to make an extragalactic origin likely. Once variability was established, follow-up observations on the Very Large Array (VLA) were used to determine the source structures. Observations were carried out at 6 and 20 cm in the A array with the resulting resolution of 0.3'' and 1.0'' respectively.

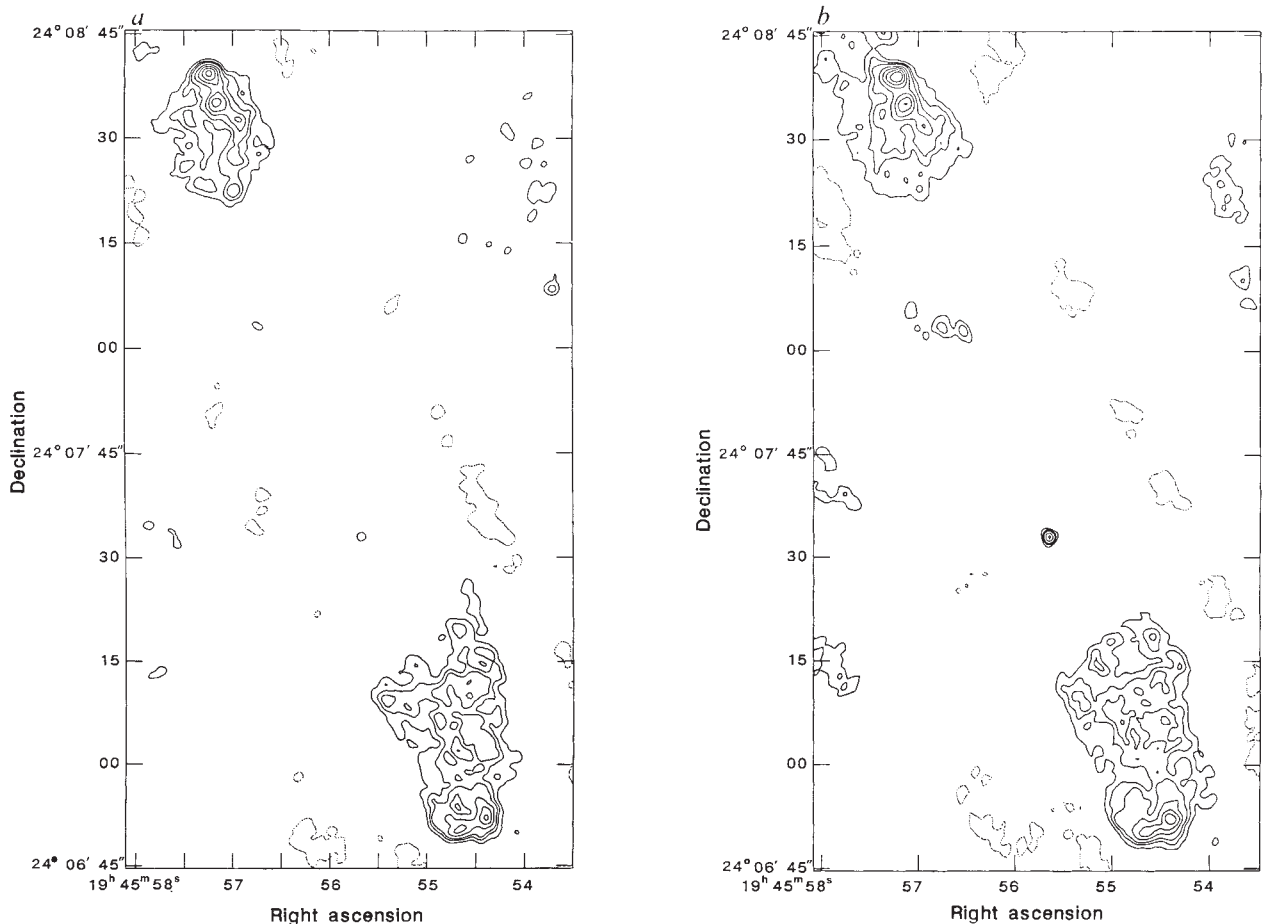


Fig. 1 *a*, The epoch-1 (January 1985) map of GT1945+241 at 20 cm. The resolution is 1.5". The core is barely visible at a signal-to-noise ratio of 5σ , where σ is the noise level (~ 0.2 mJy per beam). The peak flux is 5.1 mJy per beam. The contour levels are $4 \times 10^{-4} \times (-2.00, 2.000, 3.000, 4.000, 6.000, 8.000, 10.00, 12.00)$. With the exception of the core, the source is resolved out at 6 cm. *b*, The epoch-2 (July 1987) map of GT1945+241 at 20 cm. The core is now a 25σ direction and is ~ 5 times brighter than in the epoch-1 observations. The peak flux is 5.6 mJy per beam. The contour levels are identical in the two maps.

Ten-minute snapshots were used to reach sensitivity levels of 0.1 and 0.2 mJy respectively. Of the 31 sources mapped with the VLA, 15 are unresolved, five are doubles, four are cores with extended emission, five are triples (core+two lobes), and two are complex with no discernible cores¹². In total, 20 of these sources are core-dominated and 11 are lobe-dominated. A subset of these sources has been observed at two or more epochs with the VLA during August 1983, January 1985 and July 1987. For GT1945+241 and GT0304+575, both of which are lobe-dominated triple sources, the suspected core variability was confirmed with direct two-epoch (January 1985, July 1987) VLA flux measurements. To minimize effects resulting from the reduction procedure, great care was taken to reduce both epochs of data in identical ways. For each source the 'clean' components were restored with identical beams at the two epochs. In the case of GT0304+575 a self-calibration procedure was used to correct atmospherically induced phase errors. This procedure reduced significantly the r.m.s. noise in the final maps. Much of the extended emission of GT1945+241 was not detected, resulting in negative background levels in the region of the central core. The core fluxes had to be corrected for this effect; the correction introduces an error of ~ 0.2 mJy at both epochs at 20 cm, and of ~ 0.1 mJy at 6 cm. Combined with the errors associated with the usual r.m.s. noise (0.2 mJy at 20 cm and 0.1 mJy at 6 cm) the final errors on the core flux measurements for GT1945+241 are 0.3 mJy at 20 cm and 0.2 mJy at 6 cm for both epochs of observation.

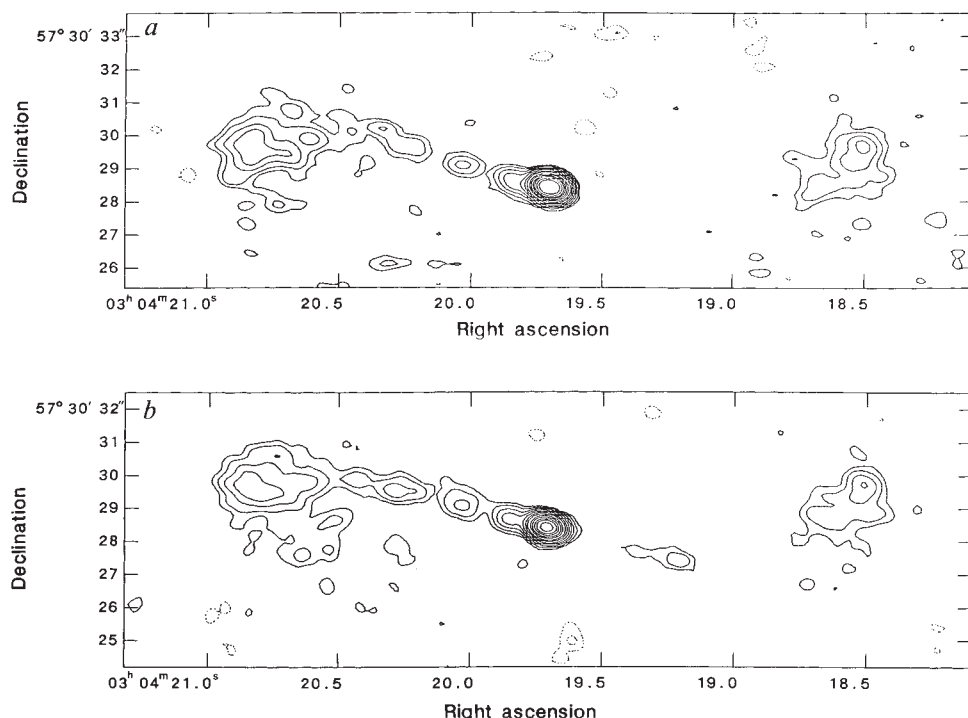
The observations, flux measurements and final errors are tabulated in Table 1. The VLA maps of the two sources at both epochs are shown Figs 1 and 2.

The first epoch observations of GT1945+241 reveal the 20-cm structure shown in Fig. 1. The nucleus exhibited an inverted spectrum with a spectral index $\alpha = +0.7$ (that is, emission intensity at frequency ν is described by $S_\nu \propto \nu^\alpha$). At that time, the core had an integrated flux density of 1.0 ± 0.3 mJy at 20 cm and 2.0 ± 0.2 mJy at 6 cm. The second-epoch observations indicated core flux densities of 5.0 ± 0.3 mJy at 20 cm and 9.4 ± 0.2 mJy at 6 cm (Fig. 1). The core brightened by a factor of five at both frequencies. GT1945+241 has a very low core fraction (ratio of core flux to total integrated flux) at both epochs and

Table 1 Summary of observations

Source	Date	Frequency (MHz)	Core flux density (mJy)
GT1945+241	1.85	1,452.4	1.0 ± 0.3
	7.87	1,452.4	5.0 ± 0.3
	1.85	4,872.6	2.0 ± 0.2
	7.87	4,872.6	9.4 ± 0.2
GT0304+575	1.85	1,452.4	46 ± 1
	7.87	1,452.4	57 ± 1
	1.85	4,872.6	92 ± 2
	7.87	4,872.6	38 ± 1

Fig. 2 *a*, The epoch-1 (January 1988) map of GT0304+575 at 6 cm. The resolution is $0.4''$. The one-sided jet geometry is evident. The peak flux density of the core is 94 mJy per beam. The contour levels are $2 \times 10^{-4} \times (-2.00, -1.00, 1.00, 2.00, 4.00, 7.00, 12.00, 20.00, 30.00, 50.00, 70.00, 100.0, 130.0, 180.0, 250.0)$. *b*, The epoch-2 (July 1987) map of GT0304+575 at 6 cm. The peak flux density of the core is halved. Although some structural variability in the jet is apparent, the variations may not be significant in these snapshot observations. The peak flux is 46 mJy per beam. The contour levels are identical in the two maps.



both frequencies of observations. Although the VLA observations resolve out much of the extended emission, lower limits on the core fraction can be placed. These range from 0.004 to 0.02 over the two epochs at 20 cm. The low core fraction precludes accurate estimates of the variability timescales from the survey data. An upper limit of about two years can be placed with the VLA observations.

In the case of GT0304+575 the integrated core flux density changed from 46 ± 1 mJy and 92 ± 2 mJy (20 cm and 6 cm respectively) to 57 ± 1 mJy and 38 ± 1 mJy. These errors are dominated by uncertainties in the absolute flux scale calibration ($\sim 2\%$). In this case the core brightened slightly at 20 cm and decreased by a factor of two at 6 cm, thus flipping the spectral index from $+0.6$ to -0.4 . The source exhibits an overall one-sided jet morphology (Fig. 2). Both the jet and the lobes are polarized at 20 cm. For GT0304+575, the core fractions are higher, averaging 0.2–0.3 over the two epochs, at both frequencies. The survey data suggest that this source varies on timescales as short as one year. Data on other lobe-dominated sources indicate variability timescales of weeks⁵, although for such cases there exists no direct VLA confirmation of variability. Although there is the suggestion of a possible feature corresponding to a ‘counterjet’ on the second-epoch map in Fig. 2, we feel it is premature to comment on its reality as it appears only as a 3σ source and may be simply a noise bump or residual sidelobe.

In the context of the beaming model, weak core sources are those whose major axes lie well away from the line of sight with little or no Doppler boosting of the emission. Furthermore, the cores of these sources should, on average, have lower degrees of variability and should vary on longer timescales. In the case of GT1945+241 the extreme variability of the core must be intrinsic and not caused by beaming (the situation for GT0304+575 is not as clear because its one-sided jet structure and higher core fraction suggest that some beaming may be possible). The fact that the core of GT1945+241 is not centred relative to the lobes suggests the possibility that it may be a variable background object. This, however, is extremely unlikely because both galactic and extragalactic variable sources are rare relative to nonvariable sources. Furthermore, the alignment of the core with the major axis of the source makes this possibility even less likely.

Indirect evidence for extreme variability exists in other weak-core sources in our sample, a good example of which is GT0545+265. Our single-epoch VLA observations indicate a lobe-dominated structure with a core flux density of 2 mJy at 6 cm. Yet the survey data indicate flux changes, at 6 cm, of 132 mJy. If the core is responsible for these variations it is varying by factors of 66 over its VLA-measured flux density. Further VLA observations are planned to measure the core changes directly.

Although a statistically significant sample of such sources is needed to test the validity of the Unified Scheme, two indirect arguments suggest that the above ‘exceptions’ to the Unified Scheme may be common. First, a full 1/3 of our sample of variable sources consists of weak-core sources (the survey data¹¹ suggest that in many cases, the sources flare on timescales of weeks). Furthermore, there is a strong selection effect that discriminates against weak-core sources in the survey. The survey is sensitive to the degree of variability in the integrated flux densities, yet weak-core sources have the lowest degrees of variability because the emission is dominated by extended non-variable structure. The fraction of 1/3 is therefore only a lower limit. Second, as the VLA data show, the cores of the weak-core sources can actually have greater relative changes in flux than the cores of the core-dominated sources⁵. This is even true between GT1945+241 and GT0304+575, as Figs 1 and 2 indicate. The statistics are clearly inadequate for generalizing these results, but if the trend persists as the sample size grows, strong constraints will be imposed on the Unified Scheme and the generality of the beaming model.

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Framework density distribution of zeolite-type tetrahedral nets

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The porosity of zeolite-type frameworks can be expressed in terms of the framework density (FD), defined as the number of tetrahedral atoms per $1,000 \text{ \AA}^3$. For the known zeolites and zeolite-type materials, FD values range from 12.5 to 20.2. Here we show that the range of the observed FD values depends on the type and relative number of the smallest rings in the tetrahedral networks. The frameworks of lowest density are those with a maximum number of 4-rings. The minimum framework density increases with the size of the smallest rings in the network. There is a clear gap in the FD values between zeolite and dense frameworks, and this also depends on the type of smallest rings present. These findings are of considerable predictive value in the important field of zeolites and molecular sieves; in particular, the minimum possible FD can be predicted for categories of frameworks defined by the smallest rings. The results of this study can serve as a guide for future efforts to synthesize low-density framework structures.

The distribution of the FD values of known framework structures as a function of the smallest ring in the structure is shown in Fig. 1. The FD values are taken from ref. 1 (excluding interrupted frameworks) except for the new zeolite beta^{2,3}, for which the FD is 15.5. The dense frameworks (indicated by + in Fig. 1) include the minerals quartz, cristobalite, tridymite, petalite, cordierite, feldspar, paracelsian, milarite, banalsite and scapolite, as well as the synthetic $\text{Cs}[\text{AlSi}_5\text{O}_{12}]$, which has an FD of 20.6 (ref. 4). For practical reasons very dense frameworks such as that of coesite (FD = 29.0) are excluded.

In the majority of networks the size of the smallest rings (minimum ring, MINR) is the same for every tetrahedral atom (T-atom). In some structures, however, the smallest ring for some T-atoms may be a 4-ring whereas for others in the same structure it is larger. This type of structure is denoted by 4+ in Fig. 1. The MINR designations 3+ and 5+ are defined in an analogous way.

A lower boundary for the FDs is clearly displayed in Fig. 1. The framework types near or on this boundary are those of CoAPO-50 (FD = 12.5/MINR = 4), faujasite (FD = 12.7/MINR = 4), zeolite beta (FD = 15.5/MINR = 4+), ferrierite (FD = 17.7/MINR = 5) and tridymite (FD = 22.6/MINR = 6). The hypothetical very open network structures postulated by Meier⁵ are also consistent with the lower boundary line (open circles in Fig. 1).

An upper limit for the FD values of presently known zeolite-type frameworks is also apparent. There is, in fact, a distinct gap of at least one FD unit in width (hatched in Fig. 1) between dense and microporous networks. The existence of this empty region indicates that zeolites are indeed a distinct class of frameworks. The lower boundary of the gap is lower for framework structures containing small rings and gradually increases with increasing ring size. It is clear from Fig. 1 that the upper limit for zeolite-type materials cannot be defined by a single FD value, as was widely presumed previously.

The framework structure of Cs-aluminosilicate, which was determined by Araki⁴, should be classified among the zeolites despite the fact that the material was reported to be anhydrous. The skeletal structure of this material is closely related to that of bikitaite. If Cs in $\text{Cs}[\text{AlSi}_5\text{O}_{12}]$ were to be replaced by a smaller cation, the material would probably be hydrated. This is similar to the isotypic framework structures of pollucite (with Cs), leucite (with K) and analcime (with Na and water).

It is also apparent in Fig. 1 that porous framework structures containing smallest rings of 5+ or 6 are unlikely to occur.

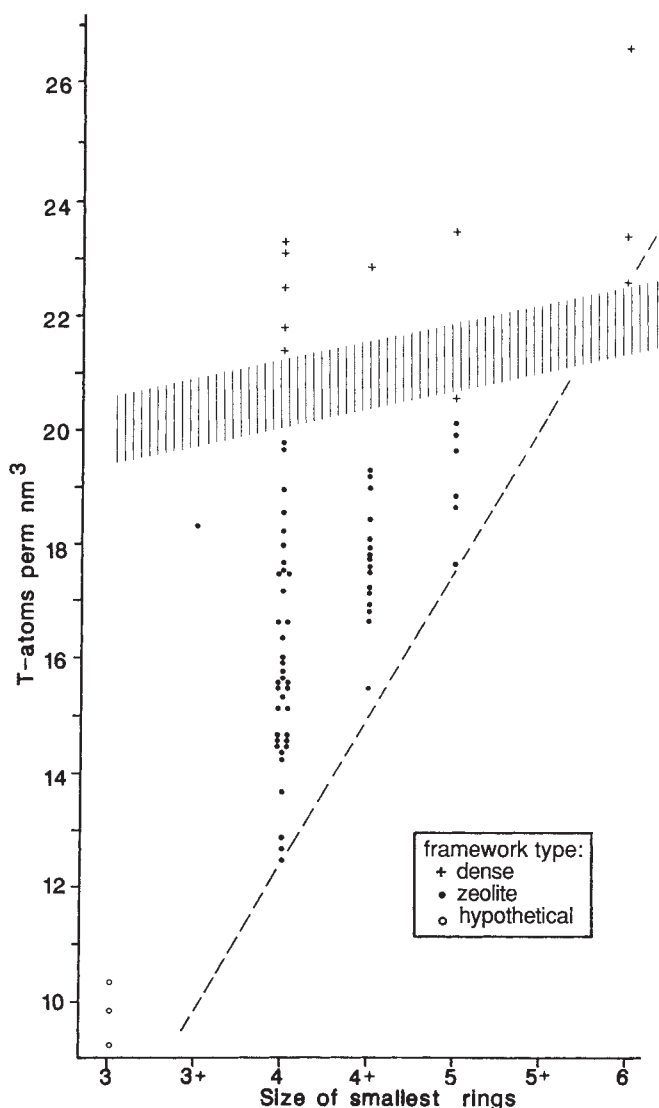


Fig. 1 Distribution of framework densities (FD) as a function of the smallest ring(s) in the network. The FD values are taken from ref. 1, with the exception of the point at FD = 15.5 (refs 2, 3). The hatched region represents the gap between dense and microporous framework types. Data points¹ (from top to bottom): 3+, LOV, 4, Feldspar, cordierite, banalsite, scapolite, paracelsian; APD, ATF, ABW, ANA, YUG, APC, LAU, GOO, BRE, AFI, SOD, CAN, ATT, LTL, MAZ, MER, AFG, PHI, LOS, LIO, ERI, PAU, OFF, EAB, LTN, LEV, KFI, GIS, GME, CHA, EDI, NAT, THO, RHO, AFS, LTA, FAU, AFY. 4+, Milarite; MTW, NON, AEL, DOH, EUO, EPI, MFI, SGT, MEL, DDR, DAC, MOR, HEU, STI, AST, beta. 5, Petalite; CAS, BIK, MTT, TON, MTN, MEP, FER. 6, Quartz, cristobalite, tridymite.

About 90% of all known zeolite-type framework structures contain 4-rings and over 60% have at least one 4-ring at every T-atom. The largest category of zeolite-like networks, in which all T-atoms are associated with a 4-ring, have FD values ranging from 12.5 to 19.8. The FD generally decreases with increasing average number of 4-rings per T-atom in the structure. Very open networks contain a maximum number of 4- and/or 3-rings per T-atom. This is consistent with the earlier predictions of Brunner⁶ which were based on a much smaller number of framework structure types.

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The formation of controlled-porosity membranes from anodically oxidized aluminium

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Synthetic membranes are used in a number of diverse applications, such as filtration^{1,2}, bioreactors^{2,3}, tissue culture⁴, analytical devices including sensors^{2,5}, and as supports for active materials^{1,5}. Narrow pore-size distribution, high pore density and thinness are often important attributes. The anodic oxidation of aluminium⁶ can produce porous films possessing these features; the anodizing voltage controls the pore size and pore density, whereas the thickness is determined by the amount of charge transferred. A major problem with this technique, however, is that the films remain attached to the aluminium, with the pore base closed by an oxide barrier layer. Here we overcome this problem by progressively reducing the anodizing voltage, thereby causing perforation of the barrier layer and separation of the film as a porous membrane.

When aluminium is anodized in certain acid electrolytes, a porous oxide develops which exhibits a remarkably uniform array of cells, each containing a cylindrical pore⁶ (Fig. 1a). During growth at constant voltage, the barrier-layer thickness remains constant because the electric field oxidizes aluminium at the metal/oxide interface and enhances chemical dissolution at the base of the pores^{6–9}. A geometric model describes how the pore size and pore density are related to the barrier-layer thickness⁶. Thus, all three parameters are controlled by the voltage. Pore sizes of 10–250 nm, pore densities of 10^{12} – 10^{15} m⁻² and film thicknesses of over 100 μ m can be achieved. The ability

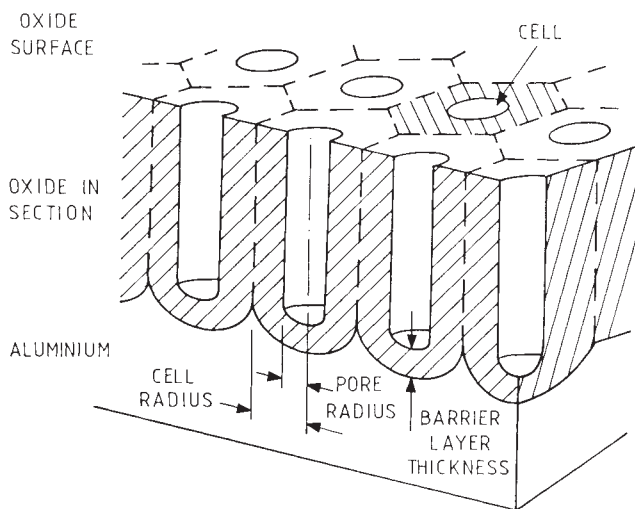


Fig. 1 Schematic diagram of a porous anodic film on aluminium, showing pores in cross-section and at a plane through the oxide, each in a hexagonal cell. During anodizing, the field is distributed across the scalloped barrier layer, separating the electrolyte within the pores from the aluminium metal.

to design porous films of pre-determined morphology makes them potentially well-suited for use as porous membranes. In order to realize this application, however, it is necessary to detach the film from the aluminium and remove the barrier layer.

The barrier-layer thickness can be reduced by lowering the anodizing voltage^{6,10}. One aim of this work was to determine whether reducing the voltage to zero causes the effective elimination of a barrier layer and thus effects film separation. But when a large voltage decrement is applied in a single step, thinning occurs only at the base of few pores^{10,11}. When the voltage is reduced, the field across the barrier layer becomes negligible. During the subsequent protracted induction period, chemical dissolution thins the barrier layer, probably non-uniformly from pore to pore, so that when the field begins to rise it is concentrated at only a few sites¹¹. Here we describe experiments in which we decrease the voltage in a series of small steps, so as

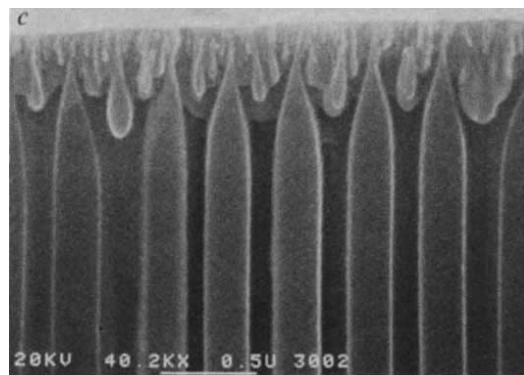
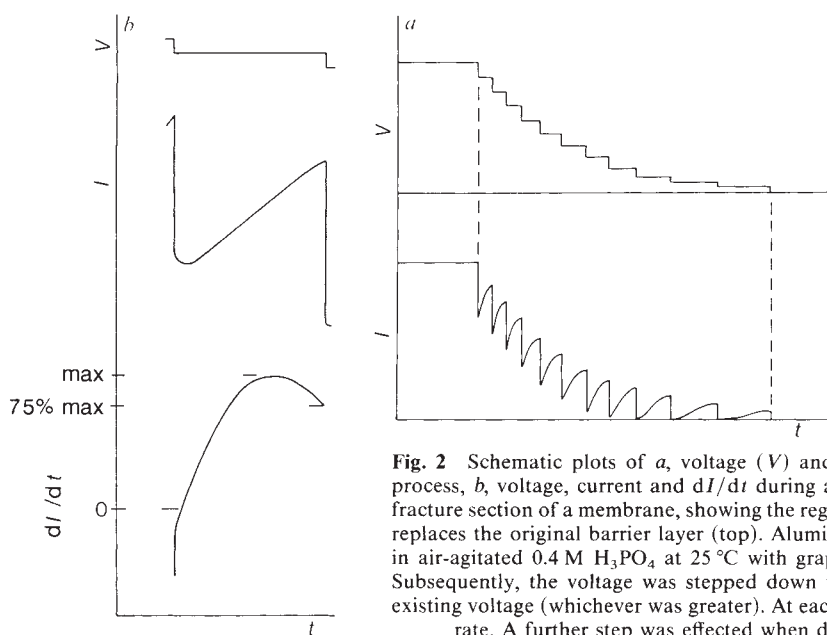


Fig. 2 Schematic plots of a, voltage (V) and current (I) against time during the voltage reduction process, b, voltage, current and dI/dt during a voltage step, and c, scanning electron micrograph of a fracture section of a membrane, showing the region adjacent to the surface where a divided pore structure replaces the original barrier layer (top). Aluminium (99.95% purity) was anodized at 160 V for 75 min in air-agitated 0.4 M H_3PO_4 at 25 °C with graphite counter-electrodes to produce a film 30 μ m thick. Subsequently, the voltage was stepped down from 160 to 0.1 V in decrements of 0.3 V or 5% of the existing voltage (whichever was greater). At each voltage step, the current fell, and then rose at a varying rate. A further step was effected when dI/dt had fallen to 75% of its positive maximum.

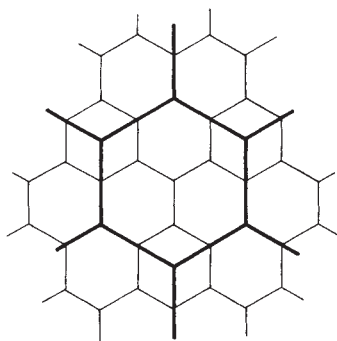


Fig. 3 Schematic representation of cell subdivision, showing how symmetry may be retained by subdivision into four.

to maintain the field at a relatively high level. It was anticipated that this would cause uniform barrier-layer thinning because, as shown by porous-film anodizing, field-assisted dissolution under high-field conditions is essentially a uniform process.

A phosphoric acid electrolyte was chosen because it permits anodization at high voltages without excessive current flow and heat evolution. Also, because they contain typically 7.5 wt% phosphate derived from the electrolyte¹², the films thus produced are relatively inert in aqueous environments¹³. The anion is distributed throughout the bulk material adjacent to the pores¹⁴, thus determining the membrane surface chemistry. Aluminium phosphate has a particularly low solubility in aqueous solution¹⁵, and phosphate absorbed onto alumina reduces the zero-point-of-charge from pH 8 to pH 9 (refs 16, 17). Electro-osmotic experiments¹⁸ have shown that the membranes described here have a negative zeta potential over a pH range of at least 3 to 10 (D. T. Hughes and W. R. Bowen, personal communication).

Monitoring the current during the voltage-reduction process revealed short induction periods and relatively high fields (Fig. 2a and b). Electron microscopy showed that essentially every pore had divided into many smaller pores, which continued to propagate as the barrier layer thinned (Fig. 2c). After each voltage decrement, the current progressively rose towards the steady value associated with the new barrier-layer thickness. When the rate of current rise had fallen to 75% of its maximum value (Fig. 2b), a further decrement was effected. The process took about 30 min, after which penetration of the barrier layer had occurred and chemical attack at the aluminium/film interface caused detachment. The membrane had an asymmetric structure: larger pores extending through the bulk of its thickness interconnected with an array of smaller pores which formed a 'skin' at that surface originally attached to the aluminium (Fig. 2c).

Inspection of electron micrographs revealed that pore subdivision produces three layers of different morphology within the skin. The geometric model⁶ was used to predict values for morphological parameters, which were compared with electron

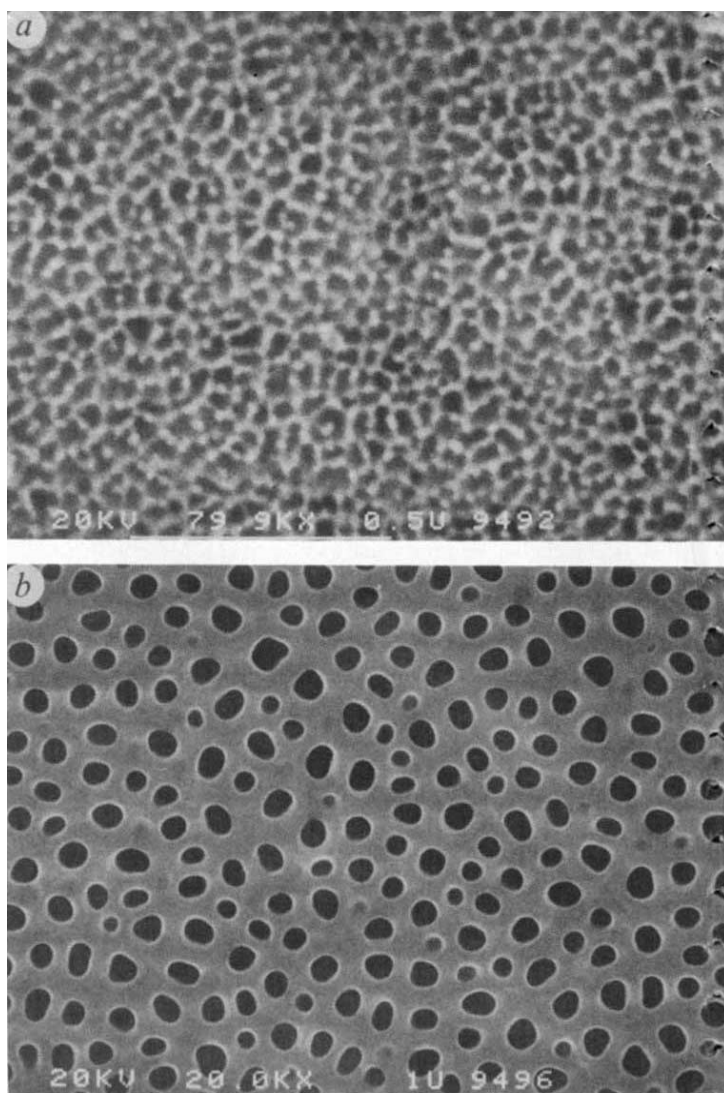


Fig. 4 Scanning electron micrographs of a, the surface of the membrane originally in contact with the aluminium metal, showing fine pores, and b, the surface of the opposite side of the membrane, after ion-beam etching, showing larger pores. Ion-beam etching (argon ions at 6 keV to 120 μ A for 20 min onto the rotating membrane at 20° incidence angle) was performed to expose the steady-state pore structure below the surface, but the apparently bimodal distribution indicates that a steady-state structure was not reached.

microscopy data of typical regions of membrane surface (Table 1 and Fig. 4). Observed values of pore size and porosity were consistently higher than those predicted, as a result of chemical dissolution in the highly acidic electrolyte. The data for the

Table 1 Comparison of theoretical and observed values for structural parameters of an anodic membrane

Voltage (V)	Calculated			Observed		
	Pore density $n_c(\text{m}^{-2})$	Pore radius $p_c(\text{nm})$	Porosity P_c	Pore density $n(\text{m}^{-2})$	Pore radius $p(\text{nm})$	Porosity P
160	6.8×10^{12}	80	0.14	1.1×10^{13}	80	0.25
80	2.7×10^{13}	40	0.14	—	—	—
40	1.1×10^{14}	20	0.14	—	—	—
20	4.4×10^{14}	10	0.14	3.8×10^{14}	15	0.31

The values and relationships¹⁹ used for the calculations were: pore radius $p_c = (0.5 \text{ nm V}^{-1}) V$, inscribed cell radius $c = (1.35 \text{ nm V}^{-1}) V$ (p_c and c in nm), pore density $n_c = 1/\pi c^2$, porosity $P_c = (p_c/c)^2$. It was assumed that each cell, and thus each pore, divides into four to maintain symmetry, as illustrated in Fig. 3. Direct observation was achieved by electron microscopy (as shown in Fig. 4), and data were obtained from areal analysis of pore images.

ion-beam-etched surface were consistent with an original pore size of 60 nm, which is typical of a lower anodizing voltage. This reflects the initial anodizing conditions, when the voltage was increased slowly from zero. Thus it appears that the geometric model provides a valid description of anodic membranes.

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Climatic significance of δD variations in a tropical tree species from India

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The climatic significance of the stable-isotope ratio of hydrogen (δD) in tree cellulose has been demonstrated using data mainly from coniferous trees that grew in temperate climates¹⁻⁴. In such regions, the mean annual δD of precipitation is linearly related to the mean annual temperature⁵, and trees thriving on precipitation preserve this temperature signal in the δD of the carbon-bound hydrogen of cellulose in their growth rings. Similar studies on tropical deciduous trees could yield useful information on climate, such as the monsoon variability over the past few centuries. However, because of the small summer-to-winter temperature contrast, tropical trees grow throughout the year and hence do not lay down well-defined growth rings (even in the few species with clear rings, it is difficult to ascertain if they are annual⁶). Also, throughout the year tropical mean surface air temperatures are $>20^\circ\text{C}$, above which the linear relation between δD of precipitation and temperature breaks down⁷. Thus there is no temperature signal in the δD of precipitation for the trees to record. In spite of these problems, we report here a significant correlation (0.7) between δD variations in two individual teak trees from the west coast of India with the amount of rainfall and mean maximum temperature, as in the case of temperate trees⁴. We propose a mechanism whereby δD of teak tree cellulose is correlated with amount of rainfall.

Teak (*Tectona grandis*, Linn. f.) was chosen because it is widely distributed in peninsular India and, unlike other tropical species, produces fairly well defined annual⁸ growth rings. The timber is used extensively for a variety of construction purposes and therefore is suitable for building long tree-ring chronologies, as timber from archaeological sites may be used for this purpose. Furthermore, it grows in well drained soils⁹ and therefore relies on rainwater for its growth. Several tree disks have been collected from the Murbad forest, Maharashtra, India ($19^\circ 14' \text{N}$, $73^\circ 24' \text{E}$, 50 m above mean sea level). They were dated using standard procedures¹⁰ and showed a good cross-match (similarity between ring width patterns of different trees) with no missing or double rings. The mean annual rainfall here ($\sim 60 \text{ km}$ north-east of Bombay) is 250 cm; 95% of it falls during the monsoon season, June to September. Relative humidity ranges from 80 to 90% during monsoons and is 70% during the rest of the year. The mean annual temperature is 27.5°C , the hottest month (May) being 6°C hotter than the coldest (January).

Teak trees in this area remain leafless during March to May. In the first two months of monsoon (June, July), the tree starts rapid leaf growth followed by shoot growth. The cloud cover

in July and August is generally 85-90% and light levels are low. Photosynthates produced during this season are used mainly for leaf expansion. The leaves grow very broad (30-60 cm across). This is followed by flowering during September and October, when the cloud cover varies from 40 to 70%. From November to February, the cloud cover is 0-2% and the temperature varies from 25 to 27°C . Photosynthesis reaches a maximum during this period, using soil moisture stored during the later monsoon (mainly August) rains. Maximum annual ring diameter growth takes place during November to February, and is followed by some production of fruit^{8,9}.

Two trees, IT-2 and IT-4, that covered a span of 64 years (from 1917 to 1980 AD) were selected. The first few rings were not analysed to avoid possible juvenile effects¹¹. Cellulose nitrate was prepared using standard procedures¹² from individual rings for the period 1920-1960 AD; these rings were relatively broad (2-5 mm). Subsequent rings (up to 1980) were too narrow ($<1 \text{ mm}$) for this procedure and were analysed in groups of two to five rings. Values of δD were determined to a precision of $\pm 2\%$ using a VG Micromass 602D mass spectrometer, and the results are reported relative to the international standard V-SMOW¹³.

To check the extent of intra-ring isotope variability, we analysed two radial sections of IT-4 rings corresponding to the years 1920-1954. The mean difference between the δD values was $2.8 \pm 3.6\%$, which was very similar to the experimental error and much smaller than the total range of variation ($\sim 30\%$). The correlation coefficient between the δD values from these two sections was ~ 0.73 . The δD values of the two trees are shown in Fig. 1a. The overall trends are similar and the common variance, calculated using the procedure outlined by Fritts⁶, is 60%. The δD values of IT-2 are, on the whole, higher by $\sim 10\%$ than those of IT-4, possibly because of the difference in the δD of soil moisture around these trees; local differences of this magnitude in a forest are not unexpected¹¹, considering that the trees were separated by a distance of $\sim 10 \text{ km}$.

The seasonal-rainfall-anomaly data for the Konkan meteorological subdivision (which includes the sampling site) were obtained from the records of the India Meteorological Department, Pune, and are shown in Fig. 1b along with the yearly mean δD values for the two trees. A positive correlation between the two is apparent. As the tree cellulose δD is also governed by climatic parameters other than rainfall, a stepwise multiple-regression analysis of the δD values, with monthly mean climatic parameters (maximum, minimum and mean temperatures, rainfall, humidity and cloudiness), was performed. This revealed that the δD of tree cellulose is significantly correlated with mean maximum temperature (T_{max}) for the period November to February and seasonal rainfall anomaly (r) for June to September:

$$\delta D = -(118 \pm 2)(0.15 \pm 0.4)r + (3.35 \pm 0.80)T_{\text{max}} \quad (1)$$

with a multiple-correlation coefficient of 0.71, significant at 0.05

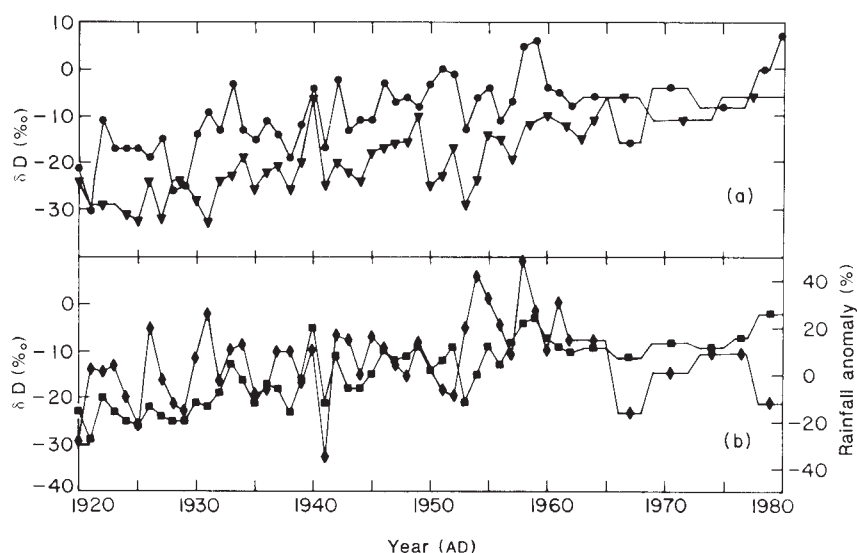


Fig. 1 *a*, δD of two trees, IT-2 (●) and IT-4 (▼) for the period 1920–1980 AD. *b*, Mean δD of the two trees (■) and annual rainfall anomaly in the Konkan sub-division (◆) from 1920 to 1980.

level, after adjusting the degrees of freedom for autocorrelation¹⁴. Because the magnitudes of variation of δD , rainfall anomaly and temperature are quite different ($\sim 30\%$, $\sim 84\%$ and 4.47°C respectively), the coefficients of r and T_{max} in equation (1) cannot be directly compared with each other⁴. All three time series can be normalized, however, by dividing the deviations from their respective means by the corresponding standard deviations, so that each of the transformed series has a mean of zero and standard deviation of unity. A multiple regression of the transformed variables yields the following equation:

$$\delta D = (1.01 \pm 0.31)r + (0.07 \pm 0.02)T_{\text{max}} \quad (2)$$

Thus it appears that there is a climatic signal in the δD of tropical teak trees, as we obtained⁴ earlier for temperate trees. This is interesting because the δD of precipitation in Bombay (from the data reported by IAEA for 13 years) is not correlated with either temperature or amount of rainfall. Therefore, the observed climatic correlation of δD of tree cellulose must be due to climatically controlled hydrogen isotope fractionation during evapotranspiration in leaves and subsequent photosynthesis⁴. Because the bulk of the photosynthates that go to make an annual ring are produced during November to February, the δD of cellulose is related to the mean maximum temperature for that period. One possible explanation for the positive correlation of δD of cellulose with the amount of rainfall is as follows. For broad-leaved deciduous trees such as teak, the length of the growing season is controlled by the availability of soil moisture. In years of low rainfall, the soil moisture reserve soon gets exhausted, and leaves wilt and drop, thereby reducing the duration of the growing season. Relative humidity decreases from $\sim 90\%$ during August to $\sim 70\%$ in February, so in such years the mean evaporative enrichment of δD of leaf water would be less because the growing season is limited to months of relatively high humidity. In years of high rainfall, the mean evaporative enrichment of δD of leaf water would be greater, because evapotranspiration could continue until the latter months, which have lower humidity; the soil moisture reserve is exhausted less rapidly. Because δD of cellulose depends on δD of the source (leaf) water, we find a positive correlation of the former with the amount of rainfall. In a broad-leaved deciduous tree such as teak, the deuterium isotope fractionation during evapotranspiration (controlled by available moisture and humidity) is much higher than the temperature-dependent biochemical isotope fractionation. Consequently, the coefficient of rainfall anomaly is more than 10 times greater than that of the temperature anomaly in equation (2).

Thus, tropical teak trees, which thrive on (monsoon) precipitation for which δD is not systematically related to air temperature

or amount of rainfall, nevertheless show a significant correlation of δD of cellulose with amount of rainfall and with the mean maximum temperature of the diameter growing season, as do coniferous trees from temperate climatic regions⁴. However, the values of the regression coefficients reported here are different from those reported by us for the latter case⁴, because of the difference in the temperature dependence of the biochemical isotope fractionation factor among species and because of the large difference in the evapotranspiration rates between broad-leaved deciduous trees (such as teak) and the needle-leaved temperate trees (such as firs). Values of δD for the teak trees are not correlated with ring width variations. This is because whereas δD of tree cellulose depends mainly on the δD of soil moisture and the ambient climate, the ring width variations are also controlled by other parameters, such as the position of the tree, its age and vigour, gravity stress, competition between trees, shading and a variety of other metabolic factors. Oxygen and carbon isotopes were not measured in the teak trees because of the uncertain role of photosynthetically released oxygen in controlling the oxygen isotope ratio of tree cellulose; also, because Bombay is an industrial area, the carbon isotope ratios will be dominated by industrial CO_2 .

We have demonstrated the potential of tropical teak trees as useful indicators of past climate. Further research in this area could lead to useful reconstruction of tropical monsoon past histories from the δD variations in teak tree chronologies.

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Influence of orbital parameters on Pleistocene loess deposition in central Alaska

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Few long proxy climatic records are available from terrestrial locations. The isotope records of Pleistocene climate change found in marine cores reflect worldwide ice-volume and temperature changes forced by variations in the Earth's orbital geometry. Comparable terrestrial records are rare. Loess (wind-deposited silt) is perhaps the closest terrestrial analogue to marine sediments in that both result from more-or-less continuous deposition of fine-grained sediment. Here we examine the hypothesis that magnetic susceptibility variations through loess sections reflect climate forcing. We statistically tested cross-correlations between high-precision, orbitally tuned marine oxygen isotope curves recording the past 250,000 yr BP, and magnetic susceptibility profiles through a thick loess section in central Alaska. Highly significant ($P > 0.0001$) correlations indicate strong, thick palaeosols formed in Arctic loess during early parts of interglacial periods corresponding to isotope stages 7 and 5. Thinner soils formed during interstadial events corresponding to early stage 7 and stage 3. Autocorrelation and time-series analysis of the loess data show spectral peaks at 125,000, 41,000 and 23,000 yr BP, showing for the first time that terrestrial orbital periodicities influenced and are recorded in proxy climatic data from terrestrial aeolian deposits at high latitudes.

Recent studies in China have suggested that bulk magnetic susceptibility varies systematically through some loess sections and can apparently be visually correlated with the well-known marine oxygen isotope curve^{1,2}, indicating that changes in the magnetic susceptibility of loess constitute a proxy climatic signal. Direct relationships between magnetic susceptibility and oxygen isotope variations have recently been demonstrated within some marine cores, where the susceptibility record is correlated with climatically controlled changes in wind intensity and aeolian sediment transport^{3,4}.

Loess is widespread in unglaciated parts of interior Alaska, where some sections are as much as 50 m thick^{5,6}. Recent sedimentological work has demonstrated that these thick sections consist largely of primary aeolian deposits⁷. We report here on stratigraphic and magnetic susceptibility studies of the important Halfway House loess section in central Alaska⁷⁻¹⁰ found at 64° 43' N, 148° 27' W, and statistically examine possible correlations between susceptibility profiles and marine proxy climatic records.

Magnetic susceptibility is a measure of the degree to which a substance perturbs a known magnetic field and is a function of the concentration and type of magnetic minerals in the sample. In this study, palaeomagnetic sediment samples were collected from loess section at 10-cm intervals, and bulk susceptibilities of 12.2 ml specimens were determined in the laboratory using a low-intensity alternating magnetic field. Non-random and systematic changes in magnetic susceptibility were found (Fig. 1). Loess in palaeosols formed during warm intervals was characterized by susceptibilities as much as an order of magnitude lower than loess deposited during colder intervals, indicating that susceptibility changes reflect climate fluctuations.

The susceptibility variations observed in loess sections, like those from marine cores^{3,4}, probably reflect climatically controlled fluctuations in average wind intensity, at least in part. By dispersing loess in small wind-tunnel experiments, we have shown that susceptibilities are rapidly modified by aeolian transport. Susceptibility variations in marine cores are commonly

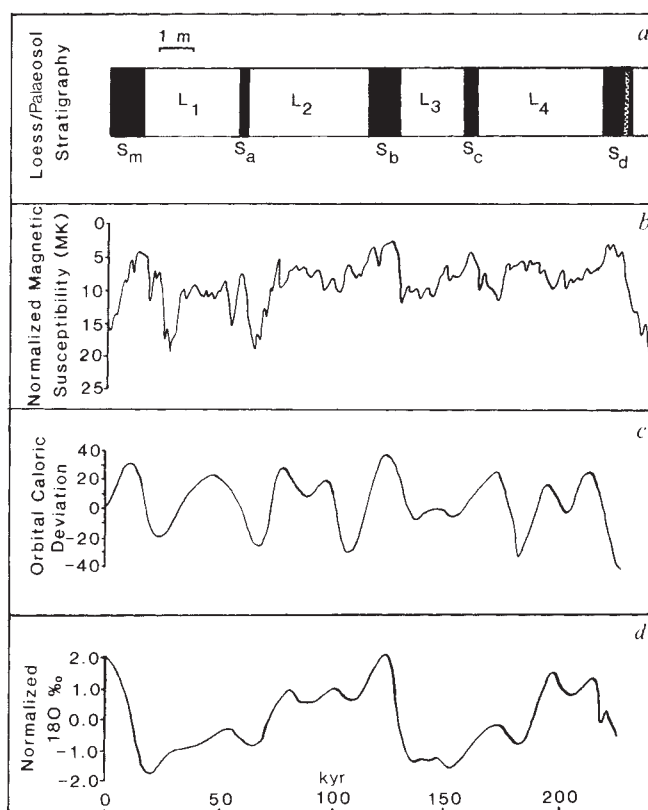


Fig. 1 a, Stratigraphy of soil and loess at the Halfway House site, central Alaska. S_m , modern soil; S_a , S_b , S_c , S_d , palaeosols. L_a , L_b , L_c , L_d , massive loess beds. Old Crow tephra is shown by a stipple pattern in S_d . b, Normalized magnetic-susceptibility variations through the loess at the Halfway House site. Sampling interval was 10 cm. c, Retrodicted insolation variations at 65° N latitude²⁸. d, Normalized and orbitally tuned marine oxygen isotope variations¹⁶.

interpreted as reflecting variations in aeolian transport of dust to marine sites^{3,4}. We extend this rationale to the interpretation of terrestrial wind-blown sediments, at the same time recognizing that pedogenic or diagenetic processes may further modify primary susceptibilities. During a single loess transportation event higher susceptibilities will be characteristic of proximal deposits, whereas more far-travelled loess will be relatively depleted in magnetic minerals; at a single spot, changes in susceptibility will reflect changes in wind velocity and competence. Similar aeolian fractionation of dense mineral phases is well-documented during dispersal of volcanic ash deposits¹¹.

We propose that average wind velocities systematically changed in central Alaska during the Pleistocene, and that the variations in wind intensities are reflected by fluctuations in the dispersal of magnetic minerals and recorded by changes in magnetic susceptibilities in loess. The pattern of low and high susceptibility in the loess data from the Halfway House section suggest that wind intensities in central Alaska were, on average, low during the Holocene and earlier interstadial and interglacial periods, and generally higher during glaciations. This is consistent with the geological data from central Alaska^{6,12}.

The age of the loess at the Halfway House site has presented a problem. More than a dozen thermoluminescence dates have been obtained from loess and volcanic ash (Old Crow tephra) near the base of the section, and reported ages have ranged from 86,000 ± 8,000 yr BP to 215,000 ± 40,000 yr BP, with different dates reflecting changes in sampling techniques and laboratory methods¹⁰. Where independent age control is available, thermoluminescence dates in loess older than 50,000–100,000 yr BP have often been shown to be biased towards underestimated

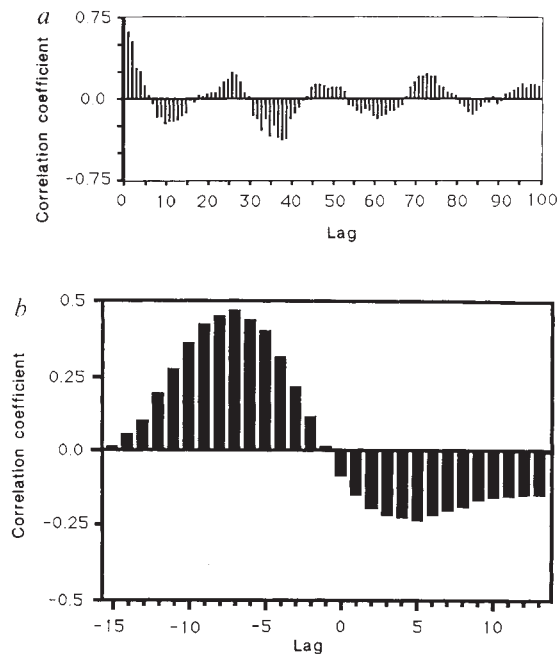


Fig. 2 *a*, Autocorrelogram of the magnetic-susceptibility sequence from the Halfway House site. Strong periodicity is indicated at a lag of 26 intervals, corresponding to 260 cm or 41,600 yr (see text). *b*, Cross-correlogram of the loess sequence and the normalized oxygen isotope data set of Martinson *et al.*²², showing the high correlation coefficient at a lag of 7 increments, corresponding to 10,000–11,000 yr. The probability of obtaining such a high correlation coefficient in two unrelated data sets is much less than 0.0001.

ages, probably because of the Debenham effect, leading some authors to suggest that all thermoluminescence dates of more than 50,000–100,000 yr BP should be considered minimum ages^{2,13,14}.

It has also been suggested that the Old Crow ash is younger than the Blake palaeomagnetic event (that is, <105–110,000 yr BP (ref. 5), but five fission track dates on the Old Crow ash have ranged from <120,000 to 156,000 ± 26,000 yr BP (refs 16, 17). Because fission track dates on Quaternary glasses, like thermoluminescence dates, often underestimate actual ages^{18,19}, and because of the lack of agreement within and between sets of thermoluminescence dates, fission track dates and palaeomagnetic correlations, we have attempted to ascertain the age of the loess and palaeosols at the Halfway House site independently.

Four palaeosols and pedocomplexes are found in the loess section (Fig. 1). Very similar soil sequences have been recognized in loess sections as far as 50 km away⁷, indicating that these palaeosols reflect climatic events. The modern soil and palaeosols *S_b* and *S_d* are as much as 1.5 m thick, and show rubification (pedogenic reddening of soil horizons) characteristic of warm conditions. Palaeosols *S_a* and *S_c* are typically only 20–80 cm thick, and do not show rubification. Two samples of charcoal were collected from a soil unit correlated with *S_a* at the Gold Hill cut near Fairbanks. Radiocarbon dates of >20,200 yr BP (1–14,936) and >37,800 yr BP (1–14,961) suggest that *S_a* and the other palaeosols formed some time beyond the limits of radiocarbon dating. All the palaeosols are locally disturbed and slumped, possibly reflecting melting of buried ground ice or ice wedges as the soil formed.

The loess intercalated between the soils is generally massive, with mottled oxidation. Some minor discontinuities, marked by changes in grain size or colour within the massive loess sections, may reflect exceptionally large dust storms or hiatuses in dust deposition. No anomalous zones of coarse sediment or sedimen-

tary structures were found, suggesting that this section consists entirely of airfall loess⁷.

A plausible correlation of the magnetic susceptibility curve from the Halfway House site with marine isotope records and Milankovitch insolation curves is suggested by visual curve fitting (Fig. 1). Correlations of the susceptibility curve seem to be supported by the position and stratigraphy of the intercalated soils, which constitute an independent palaeoclimatic record. As a first approximation, the thick rubified soils *S_b* and *S_d* resemble the modern soil, and so may record soil formation during full interglacial conditions, whereas weaker palaeosols *S_a* and *S_c* reflect briefer or less intense episodes of warming.²⁰

To test these apparent correlations, statistical comparisons²⁰ were made between the susceptibility data and recent high-resolution standard oxygen-isotope SPECMAP curves, dated by orbital tuning^{21,22}. The loess data were first detrended and ages estimated by setting the minimum susceptibility value in *S_b* to 125,000 yr BP, the age of the peak of the last interglaciation²³, and by assuming a constant sedimentation rate (orbitally tuned marine isotopic records place the maximum ¹⁸O/¹⁶O ratio at 122,560–125,190 years ago^{15,16}; ages for the last interglacial based on ²³⁰Th/²³⁴Th dating of marine terraces are 124,000 yr in New Guinea²⁴, 125,000 yr in Barbados²⁵ and 130,000 yr in Haiti²⁶). With the assumed age of *S_b* as a guide, the 1,570-cm-long loess sequence was estimated to record 250,000 years, corresponding to an average loess deposition rate of 0.006 cm yr⁻¹.

The susceptibility data appear to show periodicity. An autocorrelation test of the loess data set indicates that, independently of any assumptions of age, strong cyclicity exists with a period of ~260 cm (Fig. 2). If *S_b* is assigned the assumed age of 125,000 yr BP, then the observed 260-cm cyclicity corresponds to a period of 41,600 yr, a value quite close to that of the orbital obliquity cycle at ~42,000 yr.

A second independent statistical test of the loess data was made by interpolating the standardized marine oxygen isotope curve into the same number of intervals as the loess, and then cross-correlating between the two data sets (Fig. 2). The highest correlation coefficient ($r=0.48$) was observed when the loess data was set at lag -7 and *S_b* at 125,000 yr BP. Using Fisher's approximation, the probability of two random curves of this length having such a close match is estimated at much less than one in ten thousand, indicating that the assumed age for *S_b* is probably realistic, and that the marine and loess curves are statistically similar and correlatable. Similar curve-fitting tests for each of the conflicting radiometric dates failed to produce significant correlation coefficients (that is, for all cases $r \leq 0.19$).

We have been able to reproduce similar susceptibility curves at several other loess sections; the probability of susceptibility in multiple loess sections coincidentally correlating with SPECMAP curves is equal to the product of their individual probabilities, so that the likelihood of multiple accidental robust fits is less than one in one million. This strongly supports the interpretation that magnetic-susceptibility profiling in loess can produce proxy climatic records of the Pleistocene.

The cross-correlation calculation suggests that the normalized marine isotope record trails the magnetic susceptibility curve by ~10,000–11,000 yr (Fig. 2). This may in part reflect changes in the sedimentation rate or the presence of one or more brief hiatuses in the loess curve. But it seems likely that at least part of the lag of the marine record is real, and reflects differences in the nature of the two proxy climatic data sets. The normalized marine isotope data set is based on high-precision measurements of isotope changes in oceanic plankton, and so records the slow growth or shrinkage of continental ice sheets and concomitant isotope changes in seawater composition in response to climate change. A time lag of 9,000 ± 3,000 yr has been measured between orbital input and the obliquity cycle in some marine cores^{26,27}. The loess data, in contrast, reflect variations in average wind intensity, which might respond quite rapidly to insolation

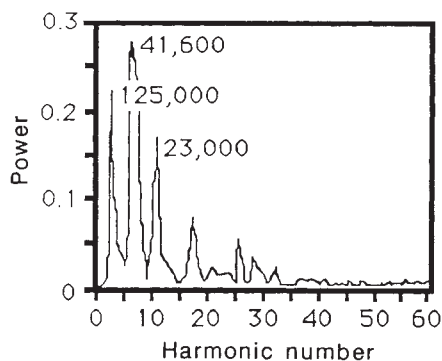


Fig. 3 Fourier analyses of the magnetic-susceptibility sequence from the Halfway House site, central Alaska. Periodicities are close to those of the Earth's orbital eccentricity, obliquity and precession.

changes. Thus a lag of the marine data set relative to the loess data is probably to be expected.

A Fourier analysis of the loess data sequence was performed to further analyse periodicity within the loess (Fig. 3). The power spectrum of the loess sequence shows three strong peaks at harmonic numbers 2, 6 and 11 (frequencies 0.013, 0.038 and 0.071 cycles per 1,570 cm, respectively). The principal peak at harmonic number 6 corresponds to the periodicity previously shown in the autocorrelogram (Fig. 2) and reflects a periodicity of 41,600 yr. Two additional peaks correspond to periods of 125,000 and 23,000 yr. The 41,600-yr period predominates, apparently reflecting the influence of changes in the angle (obliquity) of the Earth's orbital axis on loess sedimentation and climate change in central Alaska. Some models of insolation change indicate that effects resulting from obliquity changes should predominate at high latitudes, a result consistent with our data²⁸. The 125,000 and 23,000 yr peaks show lower amplitudes and apparently record the effects of orbital eccentricity (95,000–123,000 yr) and precession (23,000 yr).

Past insolation changes resulting from variations in the Earth's orbital geometry showed strong latitudinal gradients, with the timing and magnitude of retrodicted insolation change being a function of latitude²⁸. Milankovitch suggested in 1941 that summer irradiation at 65° N is critical to the pattern of ice-sheet growth and destruction and to climate change, which is the most conspicuous aspect of Earth history over the past several million years²⁹. Most modern workers agree that insolation at high northern latitudes is critical, with some choosing 65° N (refs 16, 18, 30 and 31), others 50° N (ref. 32) or even 45° N (ref. 33). The Halfway House site lies at about 65° N latitude. The spectral analyses of the susceptibility data and statistical correlations show for the first time that in some cases loess deposition is influenced by climate changes controlled by astronomical cycles. Alaskan loess contains a proxy record of climate changes at what may be the latitude critical to the growth and destruction of the Pleistocene ice sheets and the timing of worldwide late Quaternary climate change.

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¹⁴C in fractions of dissolved organic carbon in ground water

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Fundamental information is lacking on the natural dissolved organic carbon in ground water, information both necessary to understand pollutant transport and degradation and pertinent to isotope dating of ground water and reconstructions of groundwater history. Here we report, for the first time, carbon isotope ratios of fractions of natural organic compounds in ground waters isolated from the Stripa mine (Sweden) and the Milk River aquifer (Alberta, Canada). High-molecular-weight and low-molecular-weight fractions of the organic carbon were characterized and these, along with dissolved inorganic carbon, were analysed for $\delta^{13}\text{C}$ and ^{14}C . The ^{14}C results suggest that the dissolved organic carbon originates from a combination of soil organic matter and kerogen in the aquifer matrix. The high-molecular-weight fractions show a predominant soil origin, whereas the low-molecular-weight fractions are often strongly influenced by kerogen. In both systems studied, groundwater dating by traditional ^{14}C analyses of the dissolved inorganic carbon is difficult^{1–3}. The ^{14}C activities of the high-molecular-weight fractions, which were identified as fulvic acids, follow trends consistent with ^{14}C dissolved-inorganic-carbon analyses and, in some cases, provide additional information on groundwater age.

The dissolved organic carbon (DOC) in ground water is a diverse mixture of organic compounds which can come from various sources. Thurman^{4,5} has hypothesized that DOC in ground water may originate from soil through recharge, from

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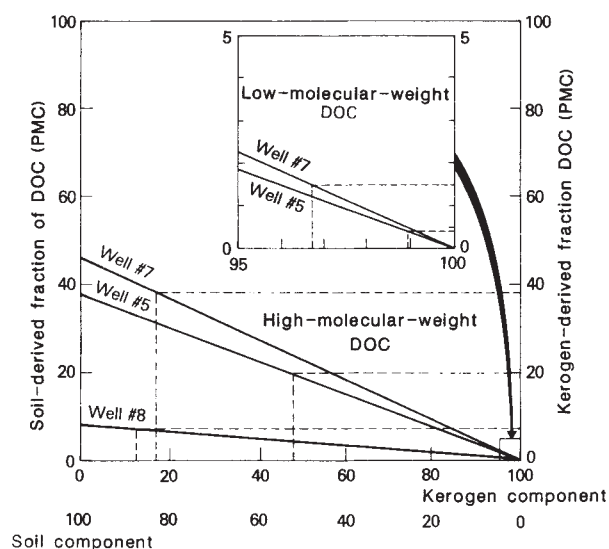


Fig. 1 Two-component mixing model for the high-molecular-weight and low-molecular-weight organic matter in the Milk River aquifer. The solid lines are the mixing lines for the organic matter in wells 5, 7 and 8. Endmembers of the mixing lines represent soil-derived DOC (DIC PMC) and kerogen-derived DOC (0 PMC). The dashed lines show the relative distribution of soil-derived versus kerogen-derived DOC and are determined by plotting the PMC of the DOC fraction. The relative distributions of low-molecular-weight compounds are shown in the inset.

kerogen embedded in the aquifer matrix, or from a combination of these two sources. Because carbon from these two sources invariably differs in age and radiocarbon content, it may be possible to distinguish the contributions by ^{14}C analyses. Analyses of ^{14}C in the DOC fraction originating from soil organic matter in the recharge zone can also provide information on groundwater ages. Here we understand groundwater age to imply 'mean apparent age'; the complexities of groundwater systems often preclude accurate assessments of age or residence time. The interpretation of groundwater age based on ^{14}C analyses of the dissolved inorganic carbon (DIC) is often complicated by carbonate reactions in an aquifer, including dissolution of solid carbonates in the aquifer matrix, precipitation of the atmospheric DIC component, isotope exchange reactions between the carbonate phases, and microbial respiration⁶⁻⁹. On the other hand, analyses of DOC ^{14}C , which is not affected by inorganic carbonate reactions, may aid in the interpretation of groundwater ages.

The Stripa mine and the Milk River aquifer are hydrologically dissimilar groundwater systems. The Stripa mine consists of a Precambrian granite dominated by fracture flow, whereas the Milk River aquifer is a Cretaceous sandstone aquifer, confined by shale beds and characterized by porous flow. The Milk River aquifer is a well defined artesian system, exemplified by a dipping aquifer with confining beds above and below¹⁰. The International Atomic Energy Agency (Vienna) sponsored the Milk River aquifer sampling trip to test methods for dating old ground water. Scientists from six countries participated in this programme. The International Stripa Project was established to study the feasibility of storing nuclear waste in granite.

The DOC was concentrated and fractionated into high-molecular-weight (HMW) and low-molecular-weight (LMW) organic compounds on a field column system. The HMW compounds (primarily fulvic acids in natural ground waters) were collected on XAD-8 resin; LMW compounds were collected on Silicalite^{11,12}, a silica-based molecular sieve with a pore size of

diameter 0.6 nm, which absorbs C_1 to $\sim\text{C}_{10}$ organic compounds^{13,14}. Although HMW and LMW were defined operationally by the sorption of the organic matter on these resins, characterization of these fractions indicates that the HMW fraction is composed primarily of fulvic acid (MW generally ranges from 500 to 950 AMU (ref. 15)), whereas the LMW fraction is composed of short-chain aliphatic hydrocarbons and substituted alcohols ($\text{MW} < 140 \text{ AMU}$)¹¹. The HMW and LMW fractions together do not represent the total DOC, but rather the two endmembers of the DOC molecular-weight continuum.

We estimate that the combination of the XAD-8 resin and Silicalite methods removes only 30–60% of the total DOC; therefore, considerable effort is required to collect DOC from ground water, which is typically present in concentrations of a few hundred $\mu\text{g l}^{-1}$. Although only a few milligrams of carbon are necessary for the accelerator mass spectrometry (AMS) analysis, $\sim 200 \text{ mg}$ of sample is required for detailed characterization of the HMW fraction. Interpretation of carbon isotope analyses without chemical characterization of the organics can produce inaccurate conclusions if the DOC originates from anthropogenic sources. For example, industrial chemicals derived from petroleum will exhibit no ^{14}C signature because of the petroleum age. To obtain one groundwater sample at the Stripa site, 1,500 l were processed, requiring two weeks of field time. Levels of natural DOC at Stripa were extremely low ($< 500 \mu\text{g l}^{-1}$), so that only a few borehole intervals were sampled.

Fractions of organic carbon were combusted to CO_2 and the $\delta^{13}\text{C}$ value was measured on a VG-602C mass spectrometer¹¹. The same CO_2 was then reduced to graphite for the AMS ^{14}C analysis^{16,17}. The DIC samples from the Milk River aquifer were precipitated with BaCl_2 , and ^{14}C was measured by scintillation counting (P. Fritz, personal communication); the Stripa DIC samples were collected in a closed system under nitrogen, acid-extracted on a vacuum line, and measured by AMS.

The isotope signature of the HMW fraction typically differs from the LMW fraction within each sample, suggesting that the separates are composed of organic compounds of predominantly different origin (Table 1). The per cent modern carbon (PMC) of the HMW DOC from the Milk River aquifer follows most closely the PMC in the DIC, suggesting that a soil origin is dominant for this fraction of the DOC. The low but measurable ^{14}C activity of the HMW DOC from well 11 indicates a maximum water age of 20,000 yr; therefore, carbonate dilution and/or isotope exchange reactions, rather than age, may account for the zero value of PMC for DIC from this well. Thus, these results show that ^{14}C measurements of specific components of the DOC may provide a more complete assessment, both of groundwater age and of biogeochemical reactions contributing to carbon concentrations and to the isotope signature of ground water, than could be obtained from DIC ^{14}C analyses alone.

The HMW and LMW fractions of the DOC could each contain a mixture of organic compounds derived from kerogen or soil. Their relative contributions to DOC for the Milk River aquifer can best be evaluated using a simple two-component mixing model (Fig. 1). The two endmembers of the mixing lines correspond to exclusively soil-derived and kerogen-derived DOC. For the former we use DIC ^{14}C values that have been corrected for carbonate reactions in the aquifer, and for the latter we assume zero per cent modern carbon content (presumably in a Cretaceous aquifer the kerogen would be older than the 45,000-yr limit to AMS ^{14}C dating).

In the case of the first (soil-derived) endmember, if the DOC originates in the soil interstitial waters of the recharge zone and is transported through the porous media, and the DIC originates from CO_2 at the land surface, then the DOC ^{14}C activity cannot be less than the DIC ^{14}C activity. DIC ^{14}C activities were corrected for carbonate reactions in the aquifer^{18,19} using the subroutine CSOTOP of the geochemical program PHREEQE; this uses the equations of Deines *et al.*²⁰ for open-system carbon-

Table 1 Radiocarbon measurements of the HMW and LWM fractions of DOC and DIC

Sample	HMW DOC		LMW DOC		Mean DIC*	
	PMC	$\delta^{13}\text{C}$	PMC	$\delta^{13}\text{C}$	PMC (age; yr BP)	$\delta^{13}\text{C}$
Milk River aquifer						
Well 5	20.0 $\pm$ 0.5	-25.5	0.4 $\pm$ 0.2	-40.0	37.5 (7,900)	-11.8
Well 7	37.6 $\pm$ 1.1	-26.0	1.5 $\pm$ 0.1	-40.2	47.0 (6,100)	-6.1
Well 8	7.2 $\pm$ 0.3	-26.0	63.5 $\pm$ 0.7	-20.2	8.0 (20,300)	-12.6
Well 11	7.3 $\pm$ 0.3	-25.8	0.6 $\pm$ 0.2	-36.5	0 (>45,000)	-16.4
Stripa Mine						
V2-2, 3 †	40.6 $\pm$ 1.0	-26.6	0.8 $\pm$ 0.2	-29.3	‡	-27.5
V2-1, 2, 3	15.3 $\pm$ 0.3	-25.0	—	—	—	-26.9
V1	—	—	3.0 $\pm$ 0.3	-34.6	14.9 $\pm$ 0.4 §	-20.5

DIC data were provided by P. Fritz (personal communication).

* The 'mean DIC' PMC and age (yr BP) for wells 5, 7 and 8 have been corrected by the authors using CSOTOP, a carbon isotope subroutine of the geochemical model PHREEQE, as discussed in the text.

† The depth below surface of the borehole intervals are as follows: V1, 758–863 m; V2-3, 898–906 m; V2-2, 958–966 m; V2-1, 967–1,230 m.

‡ PMC results from intervals V2-2 and V2-3 were 33.3 ± 0.5 and 37.9 ± 0.4 respectively for the DIC. V2-2, 3 represents a combination of these two intervals.

§ If all of the atmospheric inorganic carbon precipitates at the 300-m level of the mine, a groundwater age cannot be calculated from this ^{14}C activity.

ate reactions and those of Wigley *et al.*²¹ for closed-system carbonate reactions. The mass transfer and speciation of solutes were calculated using PHREEQE, and CSOTOP was used to calculate the carbon isotope composition of the final solution and of the precipitation/outgassing phases.

The second endmember corresponds to the case of all DOC originating from kerogen in the aquifer matrix; in this case, the organic carbon would contain no ^{14}C activity. Mixing lines for wells 5, 7 and 8 are shown as solid lines in Fig. 1. The measured ^{14}C activities of the organic fractions from each well are plotted on the mixing diagram as dashed lines. This simple mixing diagram shows that the HMW organic matter in wells 7 and 8 originates predominantly in soil (>80%). Even in well 5, almost 50% of the HMW organic matter may be derived from the soil. The LMW compounds in wells 5 and 7 (inset in Fig. 1) originate predominantly in the kerogen. Well 8 is under water-table conditions and may be receiving a small amount of infiltration from the overlying glacial till, as the ^{14}C activity in the LMW fraction seems to suggest.

Analyses of DOC ^{14}C provides additional information on DOC/DIC interactions and the groundwater age. There has been debate concerning the interpretation of DIC ^{14}C measurements at the Stripa mine. The high pH (>9) of these deep ground waters makes field collection of DIC very difficult, and hence ^{14}C activity in the DIC is often attributed to atmospheric contamination during sampling¹. Based on geochemical analyses at the Stripa site, Fritz *et al.*²² have postulated that atmospheric DIC from the recharge area probably precipitates at around 300 m depth, never reaching the deeper ground waters sampled in this study (700–1,200 m depth); in this case, it would be impossible to calculate groundwater ages based on the DIC ^{14}C activities in the samples. The presence of ^{14}C activity in the DOC, however, indicates that a relatively recent (<45,000-yr-old) component of ground water has infiltrated to this level of the mine.

Fritz *et al.*²² have also suggested that the low levels of DIC (<4 mg l⁻¹) in the deep Stripa ground waters might originate from degradation of the DOC, given the similarity between the $\delta^{13}\text{C}$ of the DIC and that of the DOC (Table 1). The ^{14}C activity of the HMW fraction from the Stripa mine is similar to the activity of the DIC, supporting their conclusions. The activity of the LMW fractions may reflect the influence of asphaltene, which has been identified in the fracture fill at the Stripa mine²³.

The ^{14}C analyses of DOC presented here show that DOC in ground water is not isotopically homogenous. Rather, different molecular-weight fractions of the DOC exhibit different isotope signatures which reflect the age, and hence location, of their

source environments. The isotope composition of these DOC fractions can be used to provide independent estimates of maximum groundwater age, to corroborate DIC age estimates, and to identify linkages between DOC and DIC pools. The approach is not without complications for groundwater dating, because the effects of mixing of source terms must be considered explicitly. We propose, however, that these analyses provide complementary information in the biogeochemical cycle of carbon in ground water which, as shown here, can provide a more complete interpretation of groundwater age and isotope composition than is possible from isotope analyses of DIC alone. In particular, DOC fractionation, studied in conjunction with isotope analyses of the DOC separates and of DIC, can highlight and resolve some of the ambiguities associated with conventional dating of ground water by DIC ^{14}C analyses alone.

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Effects of hydrogen ion and sulphate on the phosphorus cycle of a Precambrian Shield lake

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The possible deleterious effect of sulphuric acid in rain on algal abundance and productivity, by altering phosphorus recycling from sediments, has received considerable attention¹⁻³. Here I test contradictory hypotheses which predict inhibited recycling of phosphorus in response to elevated H^+ concentration, and enhanced recycling in response to elevated SO_4^{2-} . The concentrations of these ions were manipulated over two years in enclosures placed in the seasonally anoxic hypolimnion of a small Precambrian Shield lake. Seasonal releases of phosphorus from sediments in enclosures were not affected by acidification with either HCl or H_2SO_4 but increased by a factor of five in enclosures amended with sulphate salt (Na_2SO_4). The data suggests that enhanced phosphorus release was due to net alkalinity gain (alkalization) from reduction of sodium sulphate to sodium sulphide. Hence, lakes can be indirectly fertilized by addition of sulphate salts but not by addition of sulphuric acid.

Phosphorus limits the abundance and productivity of algae in most freshwaters⁴⁻⁶. Consequently, alterations in the amounts of P recycled from lake sediments by acidification of lake water could also indirectly affect algal abundance and productivity. One hypothesis that bears on how acidification from sulphuric acid in rain could affect recycling of phosphorus within lakes states that addition of H^+ inhibits P recycling because P binds to metal oxide surfaces in sediments more efficiently at higher H^+ concentrations^{1,2}. A corollary to this hypothesis is that acidification inhibits recycling of P in organic material by decomposition¹.

A second hypothesis suggests that addition of sulphate indirectly enhances recycling of P from sediments because sulphide, produced by bacterial reduction of sulphate, disrupts the interconnections between the iron and phosphorus cycles. P bound to Fe(III) oxides is released from sediments to water when iron is reduced to soluble Fe(II) in the absence of oxygen. When oxygen is reintroduced, Fe(II) is oxidized to insoluble Fe(III) oxides which co-precipitate phosphorus⁷⁻⁹. Because sulphide precipitates Fe(II), it was suggested that addition of sulphate would stimulate formation of insoluble iron sulphide species. Consequently, the P/Fe ratio released from sediments would increase and more P would escape co-precipitation when mixed with oxygenated water^{10,11}.

When both hydrogen ion and sulphate are added to aquatic systems as sulphuric acid as in acid rain, low algal productivity (oligotrophication) is predicted from addition of H^+ ; this is the reverse of the high algal productivity (eutrophication) predicted from addition of sulphate. Here I report the results of experiments in which, by altering hydrogen ion and sulphate concentrations in hypolimnetic enclosures, I test the two hypotheses and determine the interactive effects of hydrogen ion and sulphate on the release of P and Fe from the sediments of a small Canadian Shield lake. This lake is limnologically similar to the one million lakes in eastern and northern North America and Scandinavia that are most threatened by acidification.

Twelve enclosures (1 m diameter, 3 m long) made of stiff translucent polyethylene (7 mm thick) were embedded into the sediments of Lake 304 (Experimental Lakes Area (ELA), northwestern Ontario) at 4.5 m depth. The water in three replicate enclosures was amended with either NaCl, HCl, Na_2SO_4 or H_2SO_4 at the onset of thermal stratification in winter and again in summer in 1986 and 1987. Target additions were pH 4.0 for acid treatments and anion concentrations of 4 meq l^{-1} (acid

treatments were built up to this target anion concentration by adding sodium salts). A sulphate concentration of 4 meq l^{-1} is higher than mean concentrations in the acid-sensitive Pocono/Catskill region and at the ELA by 20 and 100 times respectively (ref. 12 and ELA project, unpublished data), but is only 1/14 of the concentration in sea water¹³. Excess sulphate was added because diffusive delivery of sulphate to sediments was expected to be slower in stagnant enclosures than in hypolimnia of entire lakes, and also in an effort to precipitate, as iron sulphide, many years of accumulated iron over a period of two years.

All water samples were withdrawn by pumping with a peristaltic pump through permanent sampling manifolds from fixed depths in the centre of each enclosure. Masses of each substance in the enclosures were estimated by integrating the concentration-depth profiles over the interval 10–50 cm above the sediment-water interface. The bottom waters of the enclosures remained stagnant during periods of stratification because, in summer, the enclosure walls intercepted the thermocline, and in winter, there was insufficient turbulence under ice to mix the bottom waters with the lake.

Lake 304 is a small (3.6 hectare) shallow (mean depth 3.2 m) lake located in the ELA, northwestern Ontario. The catchment is of pink granodiorite covered with very thin (0–0.5 m) glacial till and poorly developed soils¹⁴. The sediments of Lake 304 and surrounding lakes are rich in P-binding metals (Al and Fe comprise 11.6 and 6.8% respectively¹⁴) and therefore should be susceptible to short-term oligotrophication. The high iron content of the sediments suggests that they will be little susceptible to short-term eutrophication.

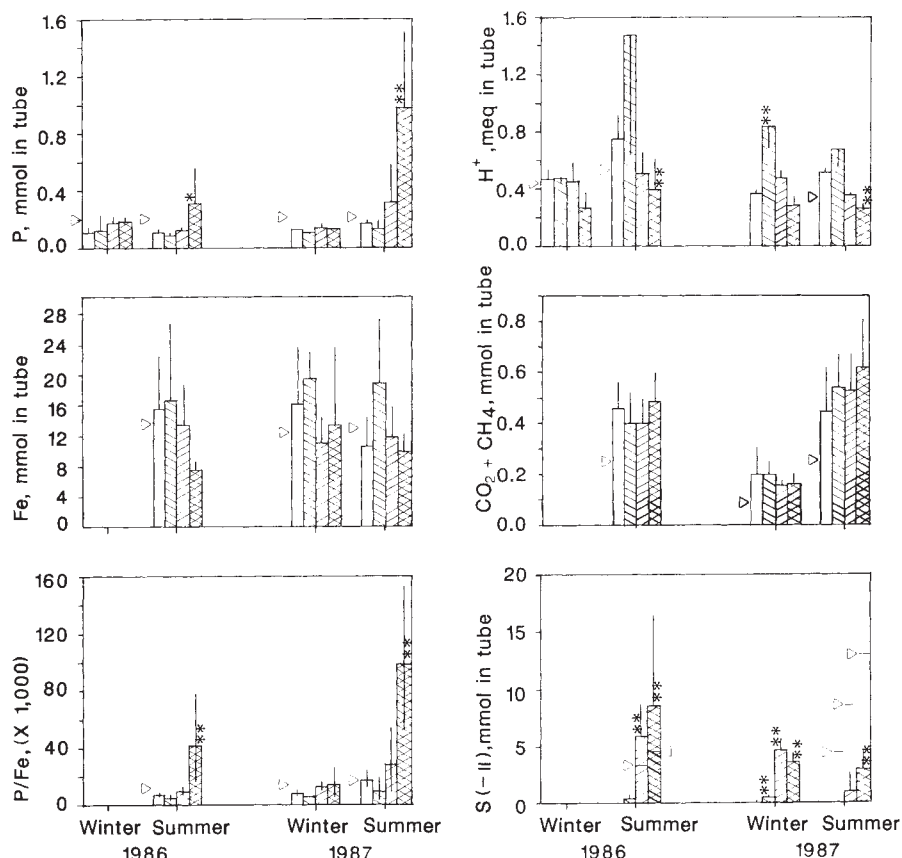
Contrary to the oligotrophication hypothesis, P release was not inhibited by addition of H^+ even though Fe and Al were abundant in Lake 304 sediments (Fig. 1). Furthermore, seasonal decomposition of organic matter was not inhibited by H^+ , because the amounts of inorganic carbon and methane (the terminal degradation products of decomposition) that accumulated in enclosures did not differ for HCl and control treatments (Fig. 1). Acid-sensitive Shield lakes are probably not oligotrophied from acidification of lake water because sediments in both acidified and unacidified Shield lakes retain P very efficiently^{5,15,16}. Acid-enhanced retention of P in drainage basins¹⁷ probably affects total P inputs to Shield lakes very little, because the largest source of P in unpolluted catchments is directly from the atmosphere¹⁸.

Contrary to the eutrophication hypothesis, the Fe cycle was apparently not disrupted by addition of Na_2SO_4 , but P releases were greatly enhanced during summer stratification. Consequently the P/Fe ratios, thought to regulate the release of P from sediments¹¹, were much higher than in control enclosures (Fig. 1). Thus, short-term data support the prediction but not the mechanism of eutrophication. During the two years of study, amounts of Fe(II) measured in the sulphate enclosures were similar to amounts measured in control enclosures. However, long-term exposure of Lake 304 sediments to elevated sulphur concentrations would probably inhibit Fe release from sediments by increasing the proportion of insoluble iron sulphides in the sediments¹⁹. Therefore the ratio of P/Fe released from sediments following long-term exposure to enhanced sulphate concentrations might be due to both enhanced P and inhibited Fe release from sediments.

The effects of hydrogen ion and sulphate on P release nullified one another when they were added together. P release was enhanced in enclosures amended with Na_2SO_4 but not those with added H_2SO_4 , even though the same amount of sulphate was added in each case. Moreover, in both cases, similar amounts of sulphide accumulated in the enclosures (Fig. 1), and a black precipitate (possibly a ferrous sulphide) was formed. Therefore neither sulphate addition followed by sulphide accumulation nor Fe precipitation appeared to regulate P and Fe release from sediments.

Fig. 1 Mean integral values ($n = 3$) of H^+ , total dissolved P, Fe(II), P/Fe, the sum of CH_4 and CO_2 , and $S(-II)$ in experimental enclosures amended with either NaCl (control) ($\square$), HCl ($\square$), H_2SO_4 ($\square$) or Na_2SO_4 ($\square$) in April (under ice) and August 1986 and 1987, within weeks of thermal destratification and mixing of the enclosures with the lake. Triangles indicate the approximate starting values in the enclosures in June/July and January when the additions were made. Mean values that are different from the control mean at the 0.05 level (pooled estimate of variation on log-transformed data) are indicated by **, and at the 0.1 level by *. Vertical lines are standard deviations, generally positive but negative where necessary to preserve scale; for the ratio P/Fe, positive and negative log-transformed standard deviations are shown.

Methods. Dissolved oxygen was measured with a calibrated oxygen probe. Dissolved inorganic carbon in gas phase equilibrated with the sample was analysed either as methane, by flame gas chromatography using a CO_2 methanizer, or directly by infrared spectrometry. CH_4 was measured in gas phase equilibrated with the sample by flame gas chromatography. The pH of samples was measured with a calibrated pH meter. Fe(II) was measured colorimetrically in unfiltered samples using the *o*-phenanthroline method²⁹. Sulphide was measured colorimetrically on unfiltered samples³⁰. Total dissolved phosphorus was measured by evaporating 20 ml of filtrate in glass vials after filtration through Whatman GF/C glass-fibre filters. Samples were ashed at 450°C for four hours, re-dissolved in 0.16 M HCl at 95°C for two hours and then analysed colorimetrically as PO_4 by the acid-molybdate method³¹.



Enhanced P release coincided with low H^+ concentrations in enclosures that contained Na_2SO_4 . Lower H^+ concentrations were probably due to sulphate-dependent alkalinity generation, caused by removal of sulphide to sediments^{20,21}, and also partly by the fact that sulphide ($pK_b = 7.1$) is a stronger base than is sulphate, ($pK_b = 12.1$ (ref. 22)) and therefore titrates H^+ . Evidently, enhanced P release was not due to enhanced decomposition of organic carbon (Fig. 1) because the amounts of inorganic carbon and methane that accumulated in enclosures were not significantly different for different treatments. Alkalinity generated in enclosures amended with H_2SO_4 was consumed by added H^+ , and hence H^+ and P concentrations approximated levels measured in control enclosures, whereas enclosures amended with Na_2SO_4 were alkalinized and enriched with P relative to control enclosures. Alkalization alone or in combination with high sulphide concentration enhanced P release in enclosures amended with Na_2SO_4 , because neither sodium (as chloride salt) nor sulphate (with hydrogen ion) affects P recycling. In subsequent experiments P concentration in the pore water of anoxic sediments from eight other ELA lakes increased in response to addition of small quantities of strong bases, including sulphide (P.J.C., manuscript in preparation).

Productivity of Canadian Shield lakes should not be affected by anthropogenic acidification of lake water by sulphuric acid. Acid-induced oligotrophication is expected to be unimportant within the Shield lakes, because I find that acidification does not alter the already very high P-binding of sediments. Eutrophication of Shield lakes by anthropogenic sulphuric acid is also expected to be unimportant, because H^+ counteracts the effect of sulphate on P release. This is consistent with observations that neither experimental addition of H_2SO_4 to acid-sensitive lakes³ nor addition of lime to anthropogenically acidified lakes²³⁻²⁵ affects P recycling from sediments.

Poorly buffered Canadian Shield lakes can be eutrophied by

addition of sulphate salts. Enhanced P recycling at high alkalinities are in accord with observed correlations reported for lakes in Wisconsin²⁶. Eutrophication of acid-insensitive lakes by anthropogenic sulphur acids is expected to be confined to lakes susceptible to alkalization. Alkalization can occur when sulphuric acid increases the weathering and importation of base cations from drainage basins into lakes and when the sulphate is reduced to generate alkalinity. Although seldom looked for, acid-induced alkalization of lakes appears to be very uncommon^{27,28}.

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Kinematics of the Alpine arc and the motion history of Adria

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The Alps have been interpreted traditionally in terms of north- or northwest-directed convergence between Africa and Europe¹, with Adria (the continental underpinnings of the Adriatic sea and northern Italy) acting as a promontory of the African continent². This has been supported by recent analyses of Africa/Europe relative plate motions^{3–5}, which indicate dominantly north-south-directed convergence during the Tertiary phase of intracontinental shortening. We show here, however, that kinematic indicators from Alpine tectonites are difficult to reconcile with this motion history (Figs 1 and 2), and are explained best by roughly west-northwest-directed Adria-Europe motion since about 70 million years ago, combined with an arc-normal pattern of motion driven by local body forces. This implies that Adria has moved independently of both Africa and Europe since at least the Late Cretaceous.

Structural indicators of the direction and sense of shear from deformed rocks in convergent orogenic belts represent particle displacement paths, and have been used to infer past plate-motion directions^{6,7}. In arcuate mountain chains such as the Alps, however, these indicators commonly show wide variations in both space and time that cannot be explained directly in terms of plate motions. Possible causes of such variations include: (1) rotation of early-formed indicators during later deformation; (2) superposition of local motions resulting from body forces generated within the region of thickened crust; (3) partitioning of the relative motion onto faults of different types within the boundary zone; and (4) the break-up of the impinging plates into lithospheric fragments capable of behaving as independent microplates (that is to say, remaining rigid and deforming only at their boundaries).

The Alps are deformed too pervasively to be treated as an assemblage of lithospheric microplates, and both Europe and Adria outside the Alps remained coherent during convergence. Limits can be placed on the effects of processes 1, 2 and 3, so we are able to use the kinematic data to show that Adria has acted as an independent microplate, and to constrain its motion. These data are summarized in Table 1 and Fig. 1. They comprise linear indicators of relative motion such as stretching lineations in mylonites, wear grooves and crystal fibres on fault planes, and (in the Jura) stylolite axes. Our compilation differs from earlier ones^{8–10} in that we show data from the entire Alpine arc, and we have included only data for which we could find good

evidence for age. The data have been separated into four age groups, based on stratigraphic evidence and on microstructural observations relating the indicators to dated metamorphic events. The four time spans are defined as follows. (1) The period of mid-Cretaceous eclogite-facies metamorphism in the Sesia and Pennine zones and the roughly coeval Barrovian metamorphism of the Austroalpine basement nappes of the Eastern Alps (~120–80 Myr old)^{11–14}. (2) The period between the mid-Cretaceous metamorphisms and the Tertiary Tauern and Lepontine Barrovian metamorphic events which peaked at around 35–40 Myr (refs 15, 16). (3) Events during and after the peak temperature of the Tertiary metamorphism (in the internal Alps), or which are post-Eocene on stratigraphic grounds (in the external zones). This is the period during which the European continental margin was shortened and imbricated, and the internal zones of the Alps were subjected to renewed thrusting and (particularly) backthrusting. (4) Mid-Miocene to Recent, during which surface deformation migrated onto the European platform (Jura and sub-Alpine chains of France).

The post-Eocene indicators (groups 3 and 4) show a striking radial pattern, swinging from roughly north in the central Alps and eastern Jura through west-northwest to south-west in the western Alpine arc. We can eliminate significant post-deformational rotation of the indicators for the youngest data (group 4): they come from weakly deformed rocks in the Jura and the French sub-Alpine chains that have been displaced a few tens of km at most, relative to stable Europe^{17,18}, and palaeomagnetic data demonstrate the absence of major rotations¹⁹. The radial pattern therefore directly reflects the kinematics of the latest stage of Alpine deformation.

The simplest explanation of simultaneous radial thrusting is that it reflects the effect of locally generated body forces. Recent analyses of the mechanics of thrust wedges show that deformation is driven by a combination of forces produced by plate convergence and those generated by gravity within the wedge itself^{10,20,21}. These analyses have not been extended to the case of convergence oblique to the trend of the mountain belt, but gravity creates pressure gradients that act in the direction of greatest surface slope, and the resulting translation will therefore lie between this direction and the plate-motion vector (PMV) (Fig. 3). Except near the terminations of a mountain chain, the surface slope will be normal to the trend of the chain, and the resultant motion vectors should lie within 90° of the PMV. The radial pattern of kinematic indicators around the Alpine arc therefore places tight constraints on the PMV: the post-15 Myr data vary from 336° (eastern Jura) to 205° (Nice arc), constraining plate motion during this period to between 246° and 295°.

The radial pattern of motion in the Alpine arc has also been interpreted in terms of plastic indentation models²². These were restricted to plane strain, however, and the predicted slip surfaces are all vertical. We do not consider this concept to be an adequate explanation of Alpine tectonics, which involved substantial crustal thickening, and where the major displacements occurred along gently dipping thrust surfaces. Numerical calculations of continental indentation show that the radial motions are driven by horizontal pressure gradients related to differences in surface elevation²³, and this appears to be confirmed by properly scaled physical models²⁴.

Indicators formed during the 40–15 Myr period (group 3) could have been rotated by later deformation, but they show virtually the same distribution as group 4 indicators, which have not been rotated significantly. Groups 3 and 4 are in fact remarkably consistent on any cross-strike traverse (Fig. 1), which suggests that they reflect the same kinematic control. Although we cannot eliminate the possibility of rotations, we can place limits on their magnitude. The continuity of geological structure in the Alps and the smooth variation in the orientation of the kinematic indicators around the arc rule out independent rotations of free-floating blocks. If continuity of structure is maintained in an oroclinal arc, any rotation must be accom-

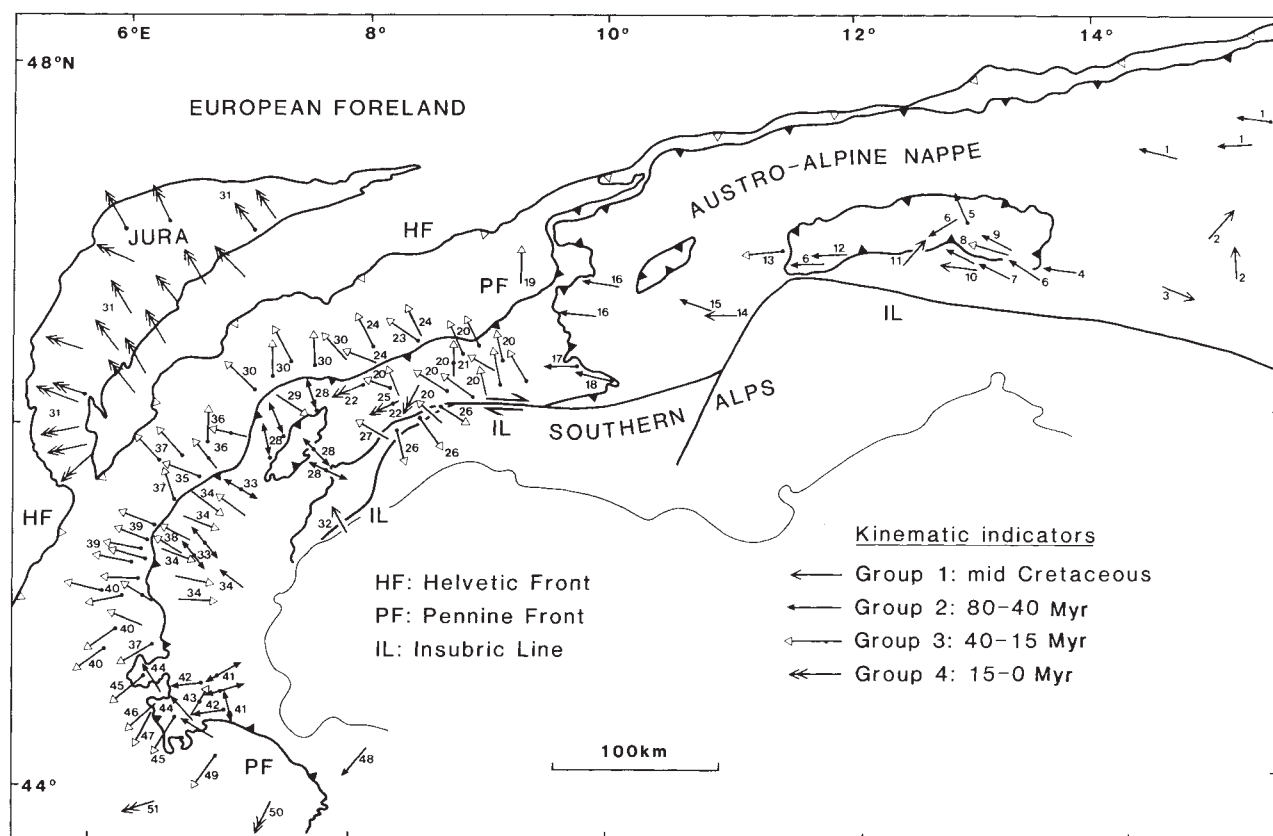


Fig. 1 Kinematic data from the Alps plotted by age. Arrow shows sense of shear. Double-headed arrows indicate that the sense is not known. The heads of arrows lie at the location of data, unless indicated by a filled circle on the arrow shaft. Note that many locations show kinematic indicators of two or more ages: these have been separated slightly on the map for clarity. For the Jura we only show data related to folding⁴². Map projection is conical orthographic.

panied by a finite strain, and the two together can be described in terms of simple shear parallel to the PMV. Rotation by 30° of a material line, initially at a high angle to the PMV, would be accompanied by a horizontal elongation of 33% at 40° to the PMV. An elongation of this magnitude, superimposed after the formation of the measured kinematic indicators, should be detectable whether it was imposed by faulting or by ductile deformation. We suggest that the lack of evidence for late horizontal elongations of this magnitude in the external zones of the Alps places an upper limit of 30° on the rotations.

The present spread of indicators in Group 3 is from 000° through west to 206° (Fig. 1). If they have not rotated, the PMV must have lain between 270° and 296° . If they have rotated by up to 30° away from the PMV, the latter must have lain between 240° and 326° . We suggest that west-northwest-directed motion is most probable during this interval (Fig. 2). The motion was probably partitioned to some extent between dextral displacement on the east-west-trending segments of the Insubric fault system²⁵ (Fig. 1) and north-south convergence in the central and southern Alps^{26,27}, but this does not affect our conclusions about the overall PMV.

Group 2 indicators (Late Cretaceous to Eocene) are, surprisingly, more consistent than the younger data: 24 of the 31 indicators shown in Fig. 1 lie between 270° and 340° , and there is little sign of a radial pattern. This is the more remarkable as many indicators of this age have been related explicitly to the exhumation of deep-seated metamorphic rocks by extension (for example, those from the Tauern window²⁸ and the Vanoise massif²⁹), a process most probably driven by body forces¹⁰. The internal zones of the Alps have experienced large deformations and displacements since Eocene time that could have significantly rotated the indicators; but the relatively consistent

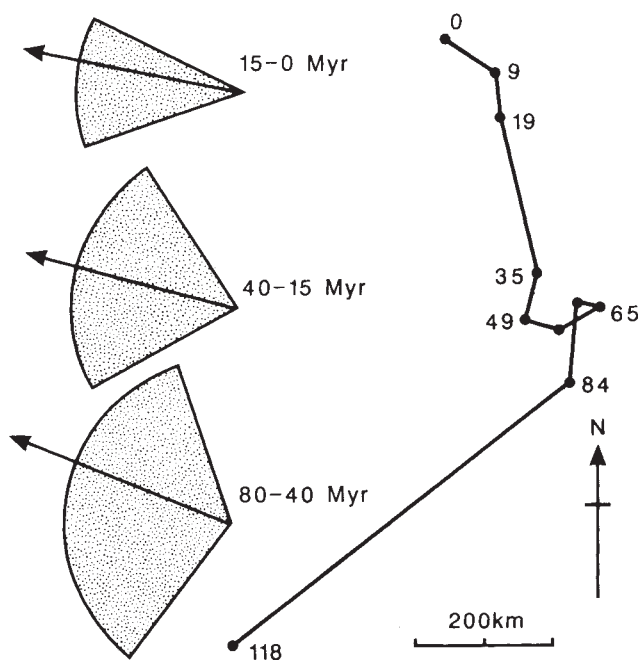


Fig. 2 Range of PMV orientations consistent with the kinematic data (shown as arcs), and our estimate of the most likely orientation (arrows), for times since the Late Cretaceous, compared with the Africa-Europe plate-motion path calculated for the location of Torino (Italy) from Africa-Europe poles redetermined by ref. 5.

Table 1 Kinematic data from the Alps

Map*	Az.†	S.o.s.‡	Age	Location	Ref.§
1	270–287	Q	Syn/post-AA	Grauacken zone	32
2	000, 045	QC	Pre/syn AA	Altkristallin	33
3	115	C	Pre/syn AA	Altkristallin	
4	280	QB	Syn/post AA	Altkristallin	J.H.B.
5	340	TC'P	Pre-T	Tauern gneiss	J.H.B.
6	240, 305	QCa	Post-HP, pre-T	Glockner nappe	J.H.B., S.W.
7	300	QPC'	Pre-T	Altkristallin	J.H.B., S.W.
8	290	Q	Post-T	Matrei Zone	S.W.
9	300	Q	Post-HP, pre-T	Glockner nappe	S.W., 16
10	280	Q	Pre/syn-AA	Altkristallin	J.H.B., S.W.
11	045	QPC'	Syn-HP	Eclogite zone	J.H.B.
12	270	B	Pre/syn-T	Glockner nappe	28
13	265	C	Post-T	Altkristallin	28
14	270	Q	Pre-AA	Altkristallin	34
15	290	Q	Syn/post-AA	Altkristallin	34
16	275, 278	PC'	Pre-L	Base of Austroalpine	J.F.D.
17	270	QC'P	Post-HP, pre-L	Margna nappe	J.H.B.
18	295	QP	Post-HP, pre-L	Platta nappe	J.H.B.
19	000	TSF	Post-Eocene	Glarus nappe	35
20	300–360	PC	Syn-L	Ticino gneiss complex	36
21	300	CC'P	Post-L	Ticino dome	36
22	210–250	CC'P	<15 Myr	Simplon dome	36
23	305	T	Post-Eocene	Gotthard massif	37
24	290–330	T	Post-Eocene	Aar massif	37
25	244	QC'	Post-20 Ma	Simplon Line	38
26	122–165	QPC	Post-L	Insubric mylonites (Sesia)	25
27	300	QPC	Post-L	Monte Rosa nappe	39
28	115–165	no	Post-HP, pre-L	Combin Zone	7
29	122	SF	Post-L	Schistes Lustrés	40
30	310–360	Ca	Post-Eocene	Helvetic nappes	41
31	228–336	T	Post mid-Mio	Jura	42
32	150	no	Syn-eclogite	Sesia Zone	43
33	115–140	no	Syn-Bg	Rutor, Vanoise, Ambin.	P.J.W., 44
34	090–125	QPC'	Post-L	Briançonnais	P.J.W., 44
35	290	T	Post-Eocene	Beaufortain	45
36	280–000	T	Post-Eocene	External massifs	46
37	238–340	T	Post-Eocene	Internal Dauphinois	47
38	295–300	TSF	Post-Eocene	Flysch, Sub-Briançonnais	M.P.
39	280–290	T	Post-Eocene	Internal Dauphinois	M.P.
40	230–300	T	Post-Eocene	Internal Dauphinois	48
41	060–345	F	Syn-Bg	Acceglio Zone	49
42	260	T	Eocene	External Briançonnais	P.C.C., 44
43	030	TCC'	Post-Eocene	External Briançonnais	P.C.C., 44
44	300–325	FF	Eocene	Parpaillon flysch	50
45	210–225		Post-Eocene	Parpaillon flysch	50
46	227	FF	Post-Eocene	Sub-Briançonnais	M.G.S.
47	206	FF	Post-Eocene	Dauphinois	M.G.S.
48	219	T	Eocene	External Briançonnais	51
49	215	T	Post-Eocene	Sub-Alpine chains	52
50	205	TPC'	Pliocene	Nice arc	53
51	250	TPC'	Pliocene	Digne arc	54

Metamorphic events. AA, Mid-Cretaceous Barrovian metamorphism in the Altkristallin. HP, Mid-Cretaceous eclogite and blueschist-facies metamorphism in internal Penninic Units. Bg, Glaucophane-schist facies metamorphism in the Briançonnais domain: post-Palaeocene on stratigraphic grounds, pre-Lepontine. T, Barrovian metamorphism in the Tauern window. L, Barrovian metamorphism in the central and western Alps.

Sense-of-shear indicators. B, Rotational porphyroblasts. C, C-S mylonitic fabric. C', Secondary shear bands in mylonites. Ca, Calcite crystallographic fabrics. F, Asymmetric folds (unreliable: sense not shown on map). FF, Small-scale structures in fault zones. P, Porphyroclast systems. Q, Quartz crystallographic fabrics. SF, Progressive rotation of fold axes with strain. T, Thrust geometry.

* See Fig. 1.

† Az: Mean trend of the directional indicator for the area shown on Fig. 1.

‡ S.o.s.: Sense-of-shear indicator.

§ Previously unpublished data collected by the authors are indicated by their initials.

|| J. Neugebauer and K. Hammerschmidt, unpublished results.

west-to-northwest trend of the data, close to the deduced PMV during the later history, argues against this. It is very unlikely that, during the Late Cretaceous to Eocene interval, the PMV lay outside the present range of Group 2 data (219–345); and the overall pattern suggests that the PMV was oriented roughly west-northwest (Fig. 2). We see little evidence for a significant change in plate motion in the Early Tertiary, as suggested by ref. 8.

Data from Group 1 (mid-Cretaceous) are too sparse and

scattered to allow useful conclusions, but they do not obviously support northward convergence at this time, as suggested by ref. 8.

The Adria-Europe motion vectors deduced from the kinematic data are compared in Fig. 2 with the Africa-Europe motion path determined from a re-analysis of Atlantic magnetic anomaly and transform-fault data³. Comparison with this or previously determined paths^{3,4} suggests that Adria must have been moving independently of Africa since at least the end of

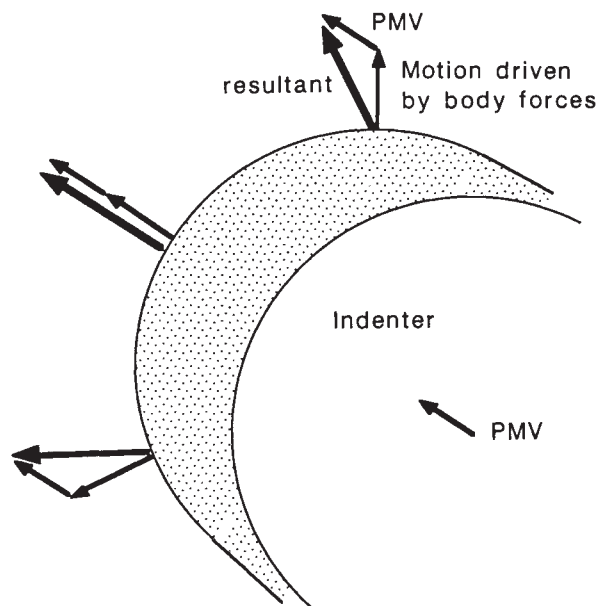


Fig. 3 Motion vectors produced by the interaction between the plate-motion vector (PMV) and body forces acting normal to the trend of a mountain belt; the latter should lie within 90° of the PMV.

the Eocene, and almost certainly since Late Cretaceous time. Independent motion of Adria is also implied by palaeomagnetic data, which suggest a counterclockwise rotation of Adria of ~15° with respect to Africa during the Tertiary³⁰, and by present-day seismicity, which indicates rotation of Adria with respect to both Africa and Europe³¹. Our conclusion, that Adria has been moving roughly west-northwest with respect to Europe throughout most of the history of Alpine orogeny, requires reassessment not only of the structure and tectonic evolution of the Alps and adjacent areas of continental Europe, but also of the other boundaries of the Adriatic microplate.

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Variable ³He/heat ratios in submarine hydrothermal systems: evidence from two plumes over the Juan de Fuca ridge

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The first vent fluid samples recovered from submarine hydrothermal systems on the Galapagos Rift¹ and at 21° N on the East Pacific Rise (EPR)² had a nearly identical ratio of ³He/heat of ~0.5 × 10⁻¹² cm³ STP cal⁻¹, even though the two hydrothermal systems were separated geographically and had widely differing fluid exit temperatures (~20 and ~350 °C, respectively)³⁻⁵. Jenkins *et al.*³ combined this ratio with independent estimates of the flux of mantle ³He through the oceans⁶, to calculate a global oceanic hydrothermal heat flux of 4.9 × 10¹⁹ cal yr⁻¹, which is in excellent agreement with geophysical estimates for this flux^{7,8}. Other investigators then combined this ³He flux with measured ratios of various chemicals in vent fluids to ³He (such as Mn/³He and Si/³He) to estimate global hydrothermal fluxes for these species^{9,10}. Here we show that ³He/heat ratios vary by over an order of magnitude between submarine hydrothermal systems, suggesting that early measurements of the ³He/heat relation are not representative of all hydrothermal systems, and that flux calculations based on the oceanic ³He flux must be undertaken with caution.

In August 1986, during a routine hydrographic survey, Baker *et al.*¹¹ detected a 700-m thick, 20-km diameter 'megaplume' over the Juan de Fuca Ridge at 44°49' N, 130°14' W (Figs 1 and 2a). At its centre, ~700 m above the sea floor, this spheroidal feature had a maximum temperature anomaly of 0.28 °C, defined by comparison of the plume conductivity-temperature-depth (CTD) profiles with the relationship of background potential temperature to potential density, which is linear for the deep water of this region^{12,13}. Although the presence of ~100-μm-sized hydrothermal mineral grains in the megaplume indicated that it was no more than a few days old at the time it was sampled, the ~2 × 10¹⁶ cal (~10¹⁷ J) of excess heat contained in this feature represents the collective output of ~100 typical black smokers venting for one year¹¹, suggesting that it was the product of a sudden, catastrophic release of heat rather than continuous hydrothermal activity. The fact that the top of the megaplume rose ~1 km above the sea floor, much higher than is observed for normal hydrothermal vent plumes, is also consistent with a sudden release of heat producing an instantaneously high buoyancy flux. Beneath the megaplume, ~250 m above the bottom, a separate deep plume was found with a

Fig. 1 Location map for the present study on the southern Juan de Fuca Ridge. The approximate outer boundary of the radially symmetric upper plume is indicated via the 0.08°C temperature anomaly contour (dashed line). Location of the vertical hydrocast (open circle) and of the horizontal tow (solid line) are also shown. Filled circles indicate the location of high-temperature vent fields previously studied using *Alvin*²⁷. Contour interval of bathymetry, 100 m.

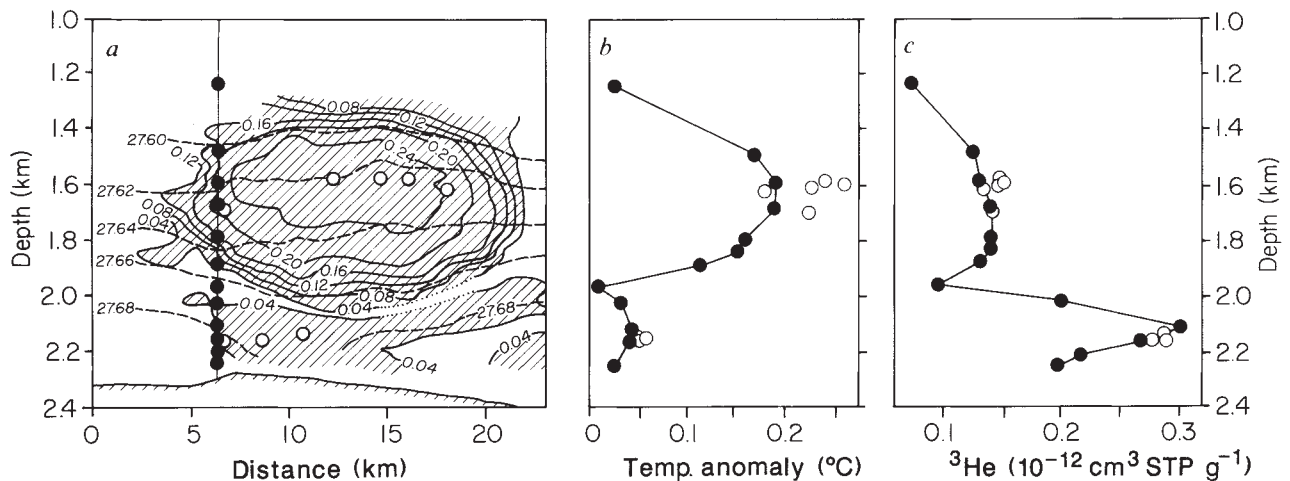
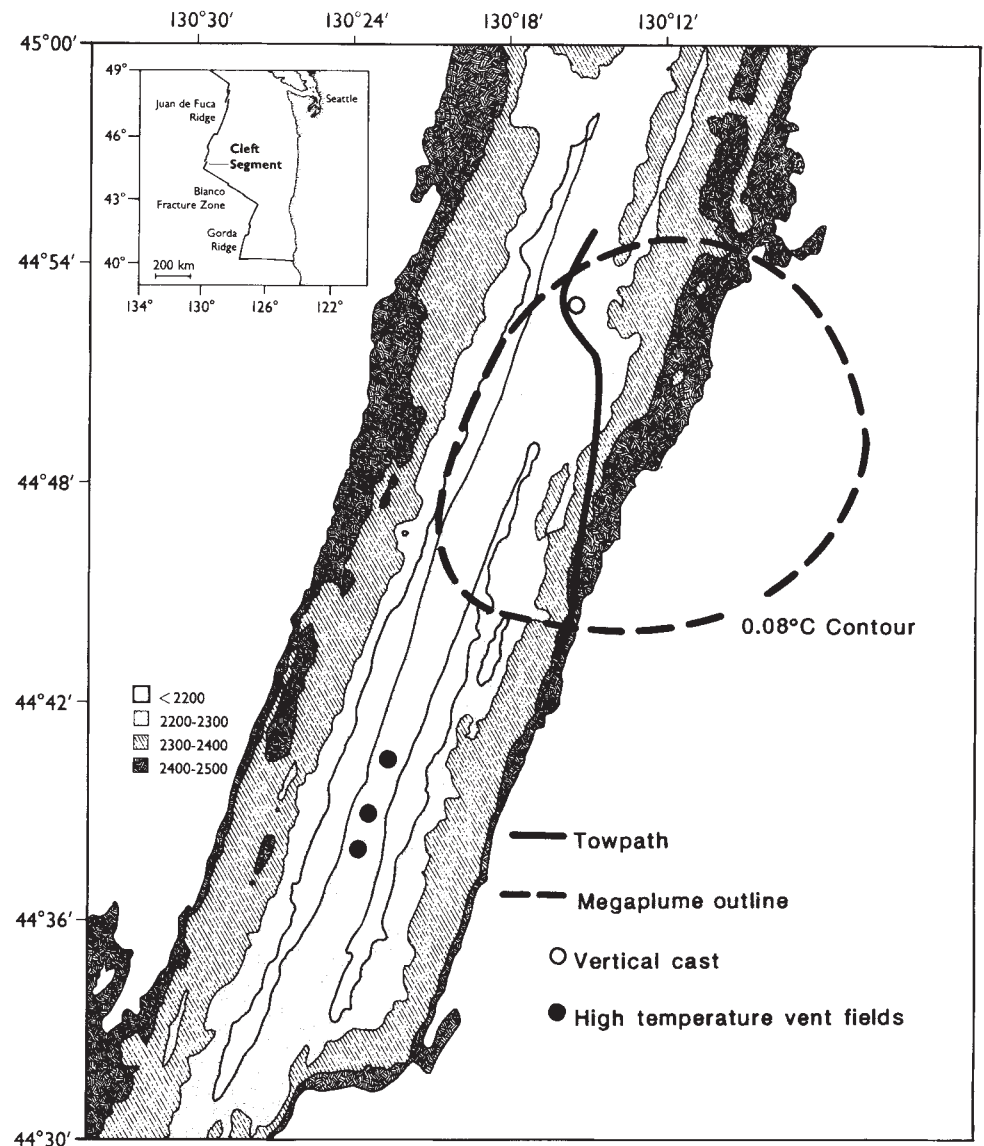
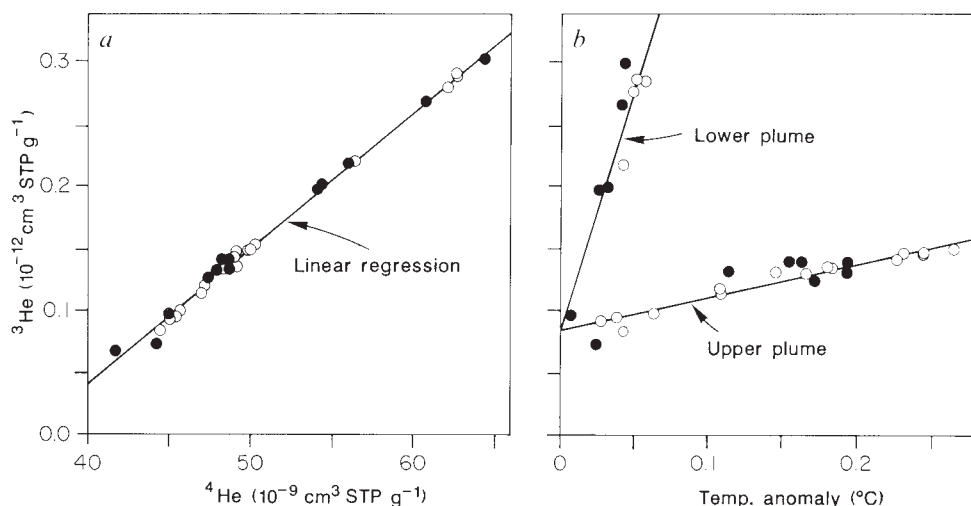


Fig. 2 *a*, North-south cross-section of temperature anomaly ($^\circ\text{C}$) along the towpath shown in Fig. 1. The temperature anomaly, which is contoured in 0.04°C increments, is the deviation of potential temperature from the normal potential temperature against potential density relationship, which is linear for the deep water of this region. The temperature anomaly is thus a measure of the excess temperature added by hydrothermal input. Dashed lines indicate surfaces of constant potential density σ_θ . Solid circles indicate water samples collected with a vertical CTD/bottle cast. Open circles indicate bottle samples collected during a horizontal tow along the towpath shown in Fig. 1. *b*, Vertical profile of temperature anomaly as a function of depth for the samples shown in Fig. 2*a*. Symbols are the same as in *a*. *c*, Vertical profile of ^3He concentration ($\text{cm}^3 \text{ STP g}^{-1}$) against depth for the samples shown in *a* and *b*. Symbols are the same as in *a*.

Fig. 3 *a*, ^3He concentration plotted against ^4He concentration for samples collected from the vertical hydrocast (solid circles) and from the horizontal tow (open circles). All of the samples from both the upper and lower plumes plot along a single line, indicating simple mixing between two end members: ambient deep sea water with $\sim 0.06 \times 10^{-12} \text{ cm}^3 \text{ STP } ^3\text{He g}^{-1}$ and $42 \times 10^{-9} \text{ cm}^3 \text{ STP } ^4\text{He g}^{-1}$, and a hydrothermal end member highly enriched in helium. The slope of the linear regression fit (solid line) indicates that the hydrothermal end member has $^3\text{He}/^4\text{He} = 1.10 \times 10^{-5}$ or $R/R_A = 7.91 \pm 0.1$, which is in very good agreement with the helium isotope signature of basalts dredged from this vicinity of the Juan de Fuca Ridge¹⁵ (here $R = ^3\text{He}/^4\text{He}$ and $R_A = R_{\text{air}} = 1.39 \times 10^{-6}$). These data



thus suggest that the helium in both plumes was derived from the local ridgecrest basalts. This figure includes some additional horizontal tow samples not plotted in Fig. 2. *b*, ^3He concentration plotted against temperature anomaly (see Fig. 1 legend) for the same data set. Symbols are the same as in *a*. Separate linear regression fits to the upper plume ($<1,950 \text{ m}$ depth) and lower plume ($>1,950 \text{ m}$ depth) data shows that the $^3\text{He}/\text{heat}$ ratio for the deep plume is ~ 15 times higher than in the upper plume; the slopes are $4.1 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$ and $0.27 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$ respectively. The actual excess heat is higher than that indicated by water column temperature anomalies because the buoyant hydrothermal plumes entrain bottom water, which is colder than the ambient water at the plume depth. However, this uncertainty does not alter the markedly different helium/heat ratios for these two plumes, as indicated by these water column measurements.

temperature anomaly of 0.06°C and an asymmetric spatial distribution which is typical of plumes produced by conventional steady-state venting into a cross-current (Fig. 2*a*)¹¹. During a second visit to this site 60 days later, this deep plume was still present, whereas no sign of the megaplume was found, lending further support to the suggestion of catastrophic and steady-state origins for the upper and lower plumes, respectively.

As discussed by Baker *et al.*¹¹, the megaplume and the deep plume had quite different chemical compositions. Although both plumes contained excess heat, dissolved silica, particulate Fe, dissolved Mn and particulate S relative to ambient seawater, the megaplume was characterized by higher Fe/Mn ratios and lower SiO_2/heat and Mn/heat ratios than the deep plume¹¹. Such differences in non-conservative properties might be expected for two different plumes, but what about conservative tracers such as helium and heat? As shown in Fig. 2, both plumes

had very strong ^3He signals, reaching maximum ^3He concentrations of $0.15 \times 10^{-12} \text{ cm}^3 \text{ STP g}^{-1}$ in the core of the megaplume and $0.30 \times 10^{-12} \text{ cm}^3 \text{ STP g}^{-1}$ in the deep plume. The corresponding $^3\text{He}/^4\text{He}$ ratios were $\delta^3\text{He} = 116\%$ and 238% in the megaplume and deep plume, respectively [$\delta^3\text{He} = 100 \times ((R_{\text{sample}}/R_{\text{air}}) - 1)$ where $R = ^3\text{He}/^4\text{He}$]. The latter value is one of the highest ^3He enrichments recorded in the open ocean. When ^3He concentration is plotted against ^4He (Fig. 3*a*), all of the samples fall on a linear mixing line which corresponds to a single hydrothermal endmember with a $^3\text{He}/^4\text{He}$ ratio 7.9 times that of atmospheric $^3\text{He}/^4\text{He}$. This value is in good agreement with measurements of helium isotopes in fresh basalt glasses from the southern Juan de Fuca Ridge¹⁵, suggesting that the helium in both plumes was extracted from local ridgecrest basalts.

The big surprise is that the $^3\text{He}/\text{heat}$ ratios in the two plumes

Table 1 Ratios of $^3\text{He}/\text{heat}$ and $^3\text{He}/^4\text{He}$ in submarine hydrothermal systems

Location	Maximum vent exit temperatures	$^3\text{He}/\text{heat}^*$ ($10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$)	$R/R_A^\dagger$	References
Galapagos Rift	$\sim 20^\circ\text{C}$	0.5	7.8	3
EPR 21°N	350°C	0.4	7.8	4, 5
EPR 13°N	$> \sim 300^\circ\text{C}$	0.7–1.4	7.5	16
Guaymas Basin	315°C	~ 0.06	7.0	17
Loihi Seamount, Hawaii	$\sim 30^\circ\text{C}$	~ 100	24.	18
Endeavour Segment, Juan de Fuca Ridge 47°57' N	400°C	1–2‡	7.9	19, 20
Taylor Vent, Axial Seamount, Juan de Fuca Ridge 46°00' N	25°C	2.2	8.1	21
Cleft Segment, Southern Juan de Fuca Ridge 44°40' N	280°C	0.5	7.7	22
Cleft Segment, Southern Juan de Fuca Ridge, megaplume: 44°48' N	—	0.14–0.3‡	7.9	This work
Cleft Segment, Southern Juan de Fuca Ridge, deep plume: 44°48' N	—	2–4‡	7.9	This work

* The $^3\text{He}/\text{heat}$ ratios have all been corrected for the specific heat of 3.2 wt% NaCl solution using the data of Bischoff and Rosenbauer²⁸. The largest correction is for the 21°N vent fluids which have $C_p = 1.53 \text{ cal g}^{-1} \text{ K}^{-1}$, at 350°C , 260 bar, compared with $1.0 \text{ cal g}^{-1} \text{ K}^{-1}$ for water at 25°C .

† Here $R = ^3\text{He}/^4\text{He}$ and $R_A = R_{\text{air}} = 1.39 \times 10^{-6}$.

‡ Values determined from measurements of the overlying hydrothermal plumes rather than by direct measurements of the hydrothermal vents. In one sense the plume measurements are more significant because they represent an average of the entire vent field output. A range of $^3\text{He}/\text{heat}$ values is reported here because entrainment of cold bottom water by the rising buoyant hydrothermal plumes introduces an uncertainty when water column temperature anomalies are converted into actual excess heat (see Speer and Rona²⁶).

differ by over an order of magnitude. As shown in Fig. 2, the megaplume has a temperature anomaly about five times that in the deep plume, whereas the ^3He signal in the megaplume is much weaker. The $^3\text{He}/\text{heat}$ ratio in the deep plume is $2\text{--}4 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$, a factor of 15 higher than in the megaplume, and a factor of seven higher than the value ($0.4 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$) measured in Galapagos Rift and 21°N EPR vent fluids. If we make the reasonable assumption that ridgecrest magmas contain ^3He and heat in constant proportion, then the differing $^3\text{He}/\text{heat}$ ratios in these two hydrothermal fluids from a single ridge segment require very different processes of formation for the two plumes. Hypotheses proposed to explain the formation of the megaplume include the sudden liberation of a large volume of hydrothermal fluid through a fissuring event¹¹, the sudden heat exchange produced by a volcanic eruption¹¹, or a catastrophic surge in the flow from the hydrothermal system¹². Of course the two plumes may be causally related, that is, the lower plume may have a larger $^3\text{He}/\text{heat}$ ratio because the mechanism which formed the megaplume preferentially extracted heat relative to helium, thereby leaving a residual system which is subsequently releasing the remainder of its volatiles. Whatever the formation mechanism, the distinct character of these two plumes indicates that the relation between ^3He and heat in submarine hydrothermal fluids is more complex than has been previously supposed.

Even without this striking example, considerable evidence already exists for wide variation in hydrothermal $^3\text{He}/\text{heat}$ ratios. As shown in Table 1, $^3\text{He}/\text{heat}$ ratios in submarine hydrothermal systems vary by two orders of magnitude, from $0.06 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$ in Guaymas Basin vents¹⁷ to $\sim 100 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$ at Loihi Seamount¹⁸. Although the Juan de Fuca megaplume is unique in terms of its physical characteristics, its $^3\text{He}/\text{heat}$ ratio is close to the Galapagos and 21°N EPR value which has been used in global hydrothermal flux calculations. On the other hand, the $^3\text{He}/\text{heat}$ ratio in the deep plume, $2\text{--}4 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$ is the highest reported for a mid-ocean ridge spreading centre system. (The value for Loihi Seamount is higher, but may not be comparable because Loihi is a hotspot rather than a spreading ridge.) The lowest $^3\text{He}/\text{heat}$ value is reported for vents in the southern trough of the Guaymas Basin, an unusual system in which the spreading rift is covered by a thick sediment pond; here the hydrothermal fluids are rich in hydrocarbons produced by thermal maturation of the organic matter in the sediments^{23,24}. It is tempting to explain these variations in $^3\text{He}/\text{heat}$ in terms of water/rock ratios: assuming that heat is extracted from basalt more rapidly than helium, the low $^3\text{He}/\text{heat}$ at Guaymas might be a consequence of high water/rock ratios caused by the confining sediment cap. In this context, the helium-rich deep plume on the southern Juan de Fuca Ridge may be the result of very low water/rock ratios.

The $^3\text{He}/\text{heat}$ ratio in the upper mantle can be estimated theoretically and compared with the values measured in submarine hydrothermal fluids. If we assume that the upper mantle is $>90\%$ degassed for helium, a figure which is a general consequence of most Earth degassing models, then it follows that virtually all of the ^4He in the upper mantle is radiogenic rather than primordial. If we further assume that radioactive decay of U and Th in the upper mantle produces $\sim 0.16 \times 10^{-6} \text{ cm}^3 \text{ STP}$ of ^4He per calorie of heat²⁵, and that the $^3\text{He}/^4\text{He}$ ratio in the upper mantle is 1.1×10^{-5} , which is the average value observed in mid-ocean ridge basalts, then the average $^3\text{He}/\text{heat}$ ratio in the upper mantle should be $\sim 2 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$, which falls within the range of values listed in Table 1 for submarine hydrothermal fluids. This estimate assumes that helium and heat are transported together in the upper mantle. If significant fractionation of helium and heat occurs in the upper mantle, such as would be produced by differential extraction of helium relative to heat during partial melting, or widely differing diffusion rates for helium and heat, then this calculation is in error.

Our theoretical estimate for the upper mantle ratio of $^3\text{He}/\text{heat}$, as well as many of the $^3\text{He}/\text{heat}$ values observed in submarine hydrothermal systems (Table 1), are considerably higher than the accepted value of $\sim 0.4 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$ that has been used to calculate global oceanic hydrothermal fluxes. This suggests that the basis for this original estimate may be in error, which is to say that either the oceanic ^3He flux is greater or the ridgecrest hydrothermal heat flux is lower than was previously supposed. The inventory of excess helium and other hydrothermal tracers in the oceans may have been underestimated by broad-scale ocean-mapping studies, which would have missed localized concentrations of hydrothermal species such as the megaplume discussed here, but this effect should make the ^3He flux low by only a small amount. Recent modelling studies suggest, however, that hydrothermal heat fluxes from axial hydrothermal venting are much lower, with the majority of the 'missing heat' being extracted by extensive off-axis circulation^{29,30}.

We conclude that heat and helium are not extracted from hot basalt at the same rate by circulating hydrothermal solutions, and that the $^3\text{He}/\text{heat}$ characteristics of the exiting hydrothermal fluids are not necessarily those of the source rock. The variations observed in $^3\text{He}/\text{heat}$ ratios in hydrothermal fluids, particularly those reported here for a single spreading ridge, suggest that the $^3\text{He}/\text{heat}$ ratio is significantly affected by the dynamic conditions within the hydrothermal system, being perhaps related to the age of the system and the water/rock ratio. These variations also suggest that global hydrothermal flux calculations based on the oceanic ^3He flux are probably only accurate to an order of magnitude. It is encouraging, however, that our theoretical estimate for the upper mantle $^3\text{He}/\text{heat}$ ratio falls in the range of values observed for submarine hydrothermal fluids, indicating that a value of $\sim 2 \times 10^{-12} \text{ cm}^3 \text{ STP cal}^{-1}$ may be a reasonable estimate for the average $^3\text{He}/\text{heat}$ signature injected into the oceans by mid-ocean ridge hydrothermal venting. Presumably a value close to this would be obtained if we could integrate the hydrothermal output from a seafloor vent field over its entire lifespan.

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Urea excretion as a strategy for survival in a fish living in a very alkaline environment

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Ammonia is toxic to all vertebrates. It can be converted to the less toxic urea, but this is a metabolically expensive process¹ found only in terrestrial vertebrates that cannot readily excrete ammonia and marine fish that use urea as an osmotic filler. Freshwater fish mostly excrete ammonia^{2,3} with only a small quantity of urea^{4,5}. It seems the ornithine cycle for urea production has been suppressed in all freshwater teleosts⁶ except for some airbreathers which, when exposed to air, increase urea synthesis via the cycle⁷. Here we show that the tilapia fish *Oreochromis alcalicus grahami*, the only fish living in Lake Magadi, an alkaline soda lake (pH = 9.6–10) in the Kenyan Rift Valley, excretes exclusively urea and has ornithine–urea cycle enzymes in its liver. A closely related species that lives in water at pH 7.1 lacks these enzymes and excretes mainly ammonia with small amounts of urea produced via uricolysis⁴. It dies within 60 min when placed in water from Lake Magadi. We suggest that urea production via the ornithine–urea cycle permits *O. a. grahami* to survive the very alkaline conditions in Lake Magadi.

Ammonia excretion by rainbow trout is impaired under alkaline conditions (pH > 9.5) (refs 8, 9). Trout are incapable of

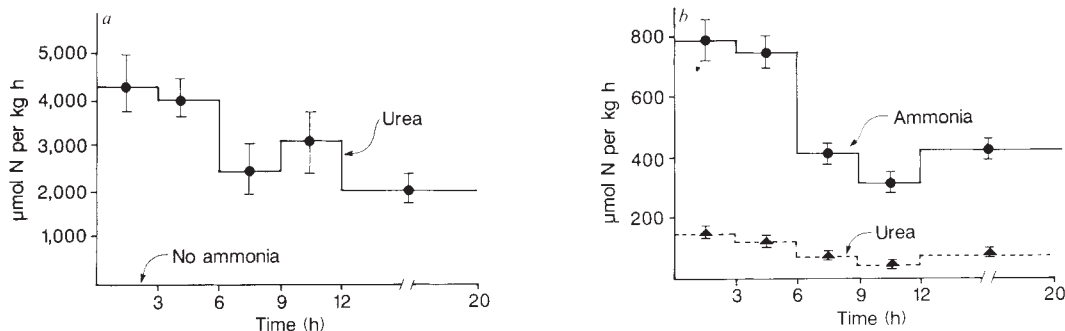
increasing urea production in the face of elevated ammonia levels, even via uricolysis³ and die when exposed to extremely alkaline conditions. The teleost, *Oreochromis alcalicus grahami* survives in very alkaline (pH 9.6–10.0) volcanic spring-fed lagoons on the margins of Lake Magadi¹⁰, because of its unique ability to excrete all nitrogenous waste as urea (Fig. 1a). These fish excrete no ammonia and no uric acid. Urea excretion rates were very high, probably reflecting the voracious feeding habits of these fish living at high temperatures (lagoon = 37 °C, laboratory = 30–34 °C). These rates declined by about 50% over 20 h, probably due to confinement stress and/or starvation. In contrast, Sagana river tilapia, *Oreochromis* sp., (probably *nilotica*, but of uncertain species because of intense cross-breeding), excretes most of its nitrogenous waste as ammonia (>80%) and only small amounts as urea (Fig. 1b), like other freshwater teleosts. These rates also declined by about 50% over 20 h, but total nitrogen excretion rates were only about 30% of that in *O. a. grahami*, reflecting the lower temperature (23 °C) of the Sagana River fish. In parallel to the differences in urea excretion, plasma urea levels were 4–5-fold higher in Magadi than Sagana tilapia, whereas plasma ammonia levels were similar (Table 1).

The liver of *O. a. grahami* contains significant levels of the ornithine–urea cycle enzymes (Table 1) in conjunction with considerable glutamine synthetase activity. By way of contrast and as in most teleost fish¹², *O. nilotica* from the Sagana river has only low or undetectable levels of the ornithine–urea cycle enzymes but much higher levels of a key uricolytic enzyme, allantoinase, compared to *O. a. grahami*.

The preferred substrate in the carbamoyl phosphate synthetase (CPS) assay was glutamine, subsequent addition of ammonia did not increase measurable enzyme activity. We conclude that *O. a. grahami* has both mitochondrial (CPS III linked to urea synthesis by the ornithine–urea cycle) and cytosolic (CPS II, involved in purine synthesis) forms of CPS, as in other fish (Mommsen and Walsh, in preparation). Magadi tilapia also have substantial activities of glutamine synthetase (GNS), an enzyme that delivers one of the substrates for CPS III catalysis, adding further support for urea synthesis via the ornithine–urea cycle in this fish. Also a high ratio of GNS/Allantoinase (6.67,

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Fig. 1 Changes in nitrogenous waste excretion with time. *a*, *O. a. grahami* (mean weight = 6.93 ± 0.70 g, 1 s.e.m., *n* = 25) in water from Lake Magadi (pH = 9.98). *b*, *Oreochromis* species from the Sagana river hatchery (mean weight = 10.76 ± 0.44 g, 1 s.e.m., *n* = 25) in Nairobi dechlorinated tapwater (pH = 7.22).



Methods. *a*, *O. a. grahami* excreted only urea at a high rate which declined with time. Experiments were carried out at ambient temperatures of between 30 and 34 °C on the balcony of the chemistry laboratory of the Magadi soda works. Water and fish were collected daily from the lagoons and hot springs in which the fish lived. Fish were placed singly in bottles containing 250 ml of Magadi water to determine excretion rates. Air was bubbled through the water having first been passed through an acid trap (0.1 M HCl) to remove any ammonia. Air leaving the bottle was then passed through a second acid trap (0.1 M HCl) to collect any volatilized ammonia, this proved to be negligible. Water samples were taken and the water renewed every three hours for 12 h and then a final sample was taken at 20 h. To exclude the possibility of bacterial ammonia or urea production simultaneous control experiments were conducted. Three groups of three bottles each contained the following: one group contained only water, a second group contained dead fish, and a third group contained recently collected fish faeces. Ammonia was added to the water at the same rate by live and dead fish and fish faeces. The dead fish and faeces did not add any urea to the water in the bottle. Thus, we concluded that the live fish, which also produced faeces during the experiment, excreted no ammonia and that any ammonia appearing in the water was produced by the faeces. *b*, Sagana River tilapia, *O. nilotica*, were collected from the Sagana tilapia hatchery and maintained in neutral Nairobi dechlorinated tapwater. Sagana River tilapia excreted more than 80% of their nitrogenous waste as ammonia and less than 20% as urea at much lower rates of total nitrogenous excretion than *O. a. grahami*, these rates also decreased with time. Excretion rates in Sagana *Oreochromis* sp. were determined at the University of Nairobi by placing the fish singly in 250 ml of Nairobi tapwater at 22 °C in aerated beakers. Water was sampled and renewed over the same schedule as in *a*. Similar experiments with dead fish and faeces demonstrated that these produce negligible ammonia and urea. There was no uric acid excretion by these fish. Ammonia was determined by the method of Verdouw, van Echten and Dekkers¹⁴, urea-N by Chaney and Marbeck¹⁵ or Crocker¹⁶, and uric acid by the method of Henry, Sobel and Kim¹⁷.

Table 1 Enzyme activities in tilapia livers

	<i>O. a. grahami</i> (Magadi tilapia)	<i>O. nilotica</i> (Sagana tilapia)
Urea cycle:		
Carbamoylphosphate synthetase:		
CPS II & III	1.24 ± 0.12 (5)	0.07 ± 0.02* (6)
CPS III	0.37 ± 0.01 (5)	0.02 ± 0.02* (6)
Ornithine carbamyl transferase (OTC)	7.32 ± 0.97 (6)	ND (6)
Arginase (ARG)	11.71 ± 0.38 (5)	13.25 ± 0.93 (6)
Intermediary metabolism:		
Glutamine synthetase (GNS)	6.14 ± 1.52 (6)	0.86 ± 0.73* (5)
Glutamate dehydrogenase (GDH)	13.87 ± 2.67 (6)	33.92 ± 5.88* (6)
Uricolytic pathway:		
Allantoicase	0.92 ± 0.15 (6)	2.65 ± 1.00 (6)
Plasma urea (mM l ⁻¹)	10.52 ± 1.06 (6)	2.32 ± 0.22* (6)
Plasma ammonia (mM l ⁻¹)	0.77 ± 0.10 (5)	1.04 ± 0.19* (6)

Enzyme activities in tilapia livers in μmol of substrate converted to product per gramme of liver tissue fresh weight in 1 min (1 h for CPS) at 22 °C under saturation conditions, $\pm$ s.e.m. (*N*). Liver were removed from freshly killed fish, frozen on dry ice and assayed within 20 d. Enzymes were assayed in 1:10 liver homogenates in HEPES buffer (50 mM, pH 7.5). CPS was assayed by a radiotracer technique¹⁸ with (CPS II & III) and without (CPS I) *N*-acetylglutamate (NAG) in the presence of glutamine. CPS II & III prefer glutamine to ammonia as a nitrogen donor, whereas CPS I is specific for ammonia. In trial experiments the addition of ammonia to the assay medium had no effect on total CPS activity and ammonia was not used in subsequent assays. OTC (pH 8.5, ref. 19), ARG (pH 8.0, ref. 19), GNS (pH 6.7; ref. 20), GDH (pH 7.4, ref. 21), allantoicase (pH 7.4, ref. 22) were assayed spectrophotometrically. ND refers to nondetectable activities. Argininosuccinate synthetase and argininosuccinate lyase activities were not measured because of limited tissue availability. These enzymes, however, are known to be present in fish generally⁶. Comparisons of enzyme activities reported here with those in the literature are of limited value because of different assay conditions and also these livers were frozen and in transit for two weeks. The livers from *O. a. grahami* and *O. nilotica*, however, were treated identically and it is this comparison on which we base our conclusions. Plasma ammonia²³ and urea¹⁴ were determined in freshly collected blood drawn by caudal puncture. * = significantly different ($P < 0.05$; Students *t*-test) for corresponding value in *O. a. grahami*.

Table 1) indicates that GNS is only involved to a minor degree in purine synthesis. CPS III activity was close to the level of detection and ornithine carbamyl transferase (OTC) could not be detected in the liver of Sagana River tilapia, indicating that urea production via the ornithine-urea cycle is insignificant in this fish. This is supported by the fact that the GNS/Allantoicase ratio is low, indicating that uricolysis is responsible for urea production in this fish. Allantoicase activity in Sagana *Oreochromis* was three times that in *O. a. grahami*, and yet urea production was less than 20% of total nitrogenous excretion. This indicates that the relative activities of enzymes may not be good indicators of the relative capacities of the two livers to produce urea by different pathways. It seems probable, however, that urea is formed only by uricolysis in *O. nilotica* and that the ornithine-urea cycle is the predominant pathway of urea production in *O. a. grahami*.

We found that *O. a. grahami* have a high tolerance to external ammonia, probably due to their high capacity to detoxify absorbed ammonia by urea production. These fish show a rapid (within a few hours) threefold increase in urea production when exposed to elevated environmental ammonia levels, whereas Sagana tilapia exhibited a negligible response to ammonia loading.

The hot spring water in which the fish lived had a total CO₂ content (bicarbonate and carbonate) of 160–200 mM compared with tilapia blood plasma level (bicarbonate) of 9.74 ± 1.15 mM

(s.e.m., $n = 9$). Blood plasma pH was 7.58 ± 0.06 ($n = 9$) when water pH was 9.98. These large bicarbonate and pH differences may cause an alkaline load for the fish. Increased bicarbonate stimulates urea production in toadfish hepatocytes¹³, and ureogenesis may play an important role in removing excess bicarbonate, reducing alkalization of the fish.

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Female choice selects for a viability-based male trait in pheasants

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Recent theory on sexual selection¹⁻⁴ suggests that females in species without paternal care choose mates by their secondary sexual characters because these indicate genotypic quality which will be transmitted to the offspring. These ideas are not yet empirically supported as data quantifying the relationship between female mate choice and female reproductive success are lacking. Only in one case, in *Colias* butterflies, has it been demonstrated unequivocally that females choose 'good genotypes' as mates⁵ and there is only one study, on *Drosophila*, demonstrating that mate choice increases one component of offspring fitness⁶. Spur length of male pheasants (*Phasianus colchicus*) correlates with various fitness-related properties⁷. We here present the first experimental field data showing that female pheasants select mates on the basis of male spur length and that female mate choice correlates with female reproductive success.

The pheasant is a precocial, sexually dimorphic species without paternal care. Male spur length is positively correlated with age, weight, size⁷ (Table 1) and with access to food as a juvenile⁸. Males with long spurs attract more females per day to their territories, and they enjoy greater reproductive success⁷. In addition, the average spur length of adult males who survive to the next season exceeds that of males who die by 2.1 mm ($P < 0.05$), indicating that spur length correlates with male viability⁷.

An isolated population of pheasants was studied in the Revinge area in southern Sweden from December 1983 to August 1987 (ref. 7). Each winter all pheasants in the study area (500 hectares) were trapped, marked, aged, biometrically measured,

Table 1 Correlations between male spur length and various male characteristics

Male characteristics (<i>N</i>)	Pearson correlation	Partial correlation (variables controlled for)
Age, A (79)	0.56† ($r_s = 0.71$)***	0.35† (B-E)
Weight, B (79)	0.58***	0.26* (A, C-E)
Wing length, C (79)	0.65***	0.46*** (A, B, D, E)
Tarsus length, D (79)	0.43***	0.15 ^{ns} (A-C, E)
Tail length, E (79)	0.34**	-0.03 ^{ns} (A-D)
Attraction of females‡ (20)	0.65**	0.61** (A-E)
Male reproductive success§ (20)	0.83***	0.81*** (A-E)

All probability tests are two-tailed: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. † Residual of dependent variable differed significantly from normality according to the Lilliefors test¹⁶. Probability levels, if given, refer to Spearman rank correlation (r_s). ‡ Average number of females per day observed less than 100 m from the male during the breeding season (April-June). § Computed from estimated paternity of successfully hatched chicks. ns, Not significant.

and released into an outdoor enclosure in the centre of the study area where the pheasants' agonistic encounters were recorded to estimate social dominance⁷. On the first weekday of April all pheasants were simultaneously released from the enclosure and radiotracking gave information on territorial establishments, female proximity to males, mortality, nesting attempts and hatching success.

After release the females visited several males before they finally settled in a territory^{7,9}. Females selected primarily males that were old, and had long spurs and wings (Table 2). Partial regression reveals that, controlling for age and wing length, spur length is a significant predictor of female mate choice, whereas controlling for spur length, neither age nor wing length is important (Table 2). Females may select mates on the basis of male territory quality¹⁰, but we found no correlation between the number of females selecting a given male and the quality of his territory (Table 2). Territory quality is a complex variable that is difficult to assess¹¹ without experimental manipulations¹⁰. We feel that our measurement of habitat quality (defined in Table 2) is supported by a significant correlation between a female's reproductive success (measured as the number of hatched chicks) and her territory quality ($P < 0.05$, Fig. 1a). These data suggest that male spur length is the most important predictor of female mate choice.

Male spur length was manipulated in an experiment performed between December 1987 and June 1988. Table 3 shows that the average number of females per day observed in a male's vicinity (<100 m, compare with Table 2) differed significantly between the shortened, control, and elongated male groups in each of the three breeding months (April-June) but the difference was greatest in May (Table 3) when egg-laying and mating are most intense⁷. The averages of male age, wing length, and original spur length did not differ between the three experimental groups. The results in Table 3 strongly indicate that variables other than spur length are not the actual targets of the female preference, but spur length itself seems to be all that matters.

One could argue that the effect of spur length is explained by intrasexual selection and that males use their spurs in dominance interactions¹². There are two observations that cast doubt on this possibility. First, our experiment with elongated and shortened spurs did not reveal any effect of spur length on male dominance in the enclosure (Table 3). In only one case out of 20 male-male interactions observed in the field during the experimental year (1988) did the outcome differ from that observed in the enclosure. This indicates that male dominance in the enclosure reflects the situation in the wild. Second, data from 1984-1987 (ref. 7) show that male dominance did not correlate with spur length, attraction of females, or male reproductive success. Instead, male dominance correlated with weight ($r = 0.35$) and tail length ($r = 0.25$, $N = 79$, two-tailed $P < 0.01$ and < 0.05 , respectively). The same pattern applied during the experimental year: partial correlation compensating for manipulated spur length was $r = 0.51$ (dominance-weight, $N = 21$, $P <$

0.01) and $r = 0.46$ (dominance-tail length, $N = 21$, $P < 0.05$). Hence, male spur length is less important for male dominance than weight and tail length, indicating that the demonstrated effect of male spur length on female mate choice does not result from intrasexual selection.

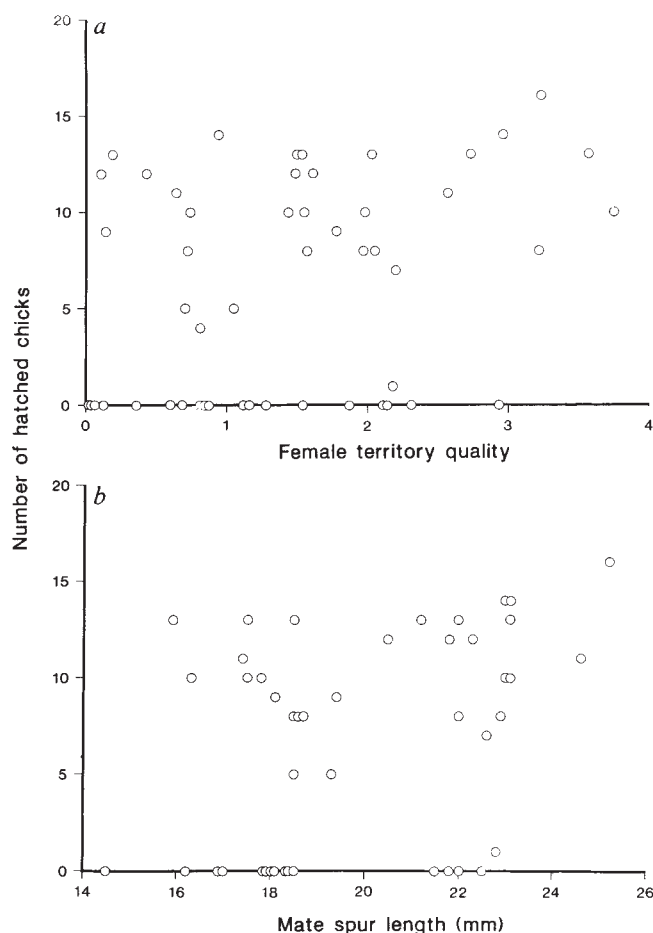


Fig. 1 Female reproductive success measured as the number of chicks that each female hatched during a breeding season (data are from 1984-1987) in relation to: (a) her territory quality (defined as in Table 2). Parametric correlation analysis ($r = 0.31$) gave a residual that differed significantly from normality¹⁶. Probability test therefore refers to non-parametric statistics, $r_s = 0.28$, $N = 51$, two-tailed $P < 0.05$; (b) the spur length of her mate (defined as in Table 2), $r = 0.36$, $N = 45$, two-tailed $P < 0.02$. A stepwise multiple regression analysis ($P < 0.05$ to enter and $P > 0.10$ to remove) with female age, weight, size (wing-, tarsus-, and tail-length), dominance¹⁹⁻²¹, territory quality, and the mate's spur length as potential predictor variables included the mate's spur length as the only significant predictor of female reproductive success.

Table 2 Differences between finally mated and unmated males in 1986–1987 and correlations between the male characters and the number of females choosing the male as final mate

Male characteristics	Mated males $N = 18$ $\bar{x}$ (s.d.)	Unmated males $N = 15$ $\bar{x}$ (s.d.)	Pearson correlation $N = 33$	Partial correlation (variables controlled for)
Age (year)	2.0 (1.0)	1.3 (0.8)†*	0.28† ($r_s = 0.43$)*	0.12† (spur length)
Spur length (mm)	19.5 (2.7)	16.7 (2.5)§**	0.61***	0.48** (age and wing length)
Wing length (mm)	217 (5)	213 (6)§*	0.40*	0.02 ^{ns} (spur length)
Territory quality	1.38 (0.96)	1.58 (1.32)‡ ^{ns}	0.08† ($r_s = 0.02$) ^{ns}	−0.05† (spur length)

All probability tests are two-tailed: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. † Residual of dependent variable differed significantly from normality¹⁶. Probability levels, if given, refer to Spearman rank correlation (r_s). ‡ Mann-Whitney U -test; § t -test. A female's final mate was defined as the male in whose vicinity (<100 m; the radius of the average male territory was 170 m) the female was observed (by radiotelemetry) most frequently during her latest territorial establishment of the breeding season (that is before 1 July). Data from 1984 and 1985 are excluded as, due to radiotransmitter failure, too few (<70%) of the females' final mate choice could be established. Data from males that died before the 1 July are also excluded. We also measured male weight, tarsus length, bill length, tail length and dominance. None of these characters differed between mated and unmated males. Spur length measurements include the diameter of the tarsus. Habitat quality was estimated using data from a previous pheasant study¹⁷ in our study area. The habitat distribution of the 29 successfully hatched pheasant nests from this previous study¹⁷ was compared with the overall habitat distribution of the study area which yielded the following quality indices: grass meadow (dominated by *Deschampsia caespitosa*) (4.24), tall herb meadow (3.00), mesic and grazed fields (dominated by *Dactylis glomerata*) (0.81), spruce plantations (0.74), and dry grass fields (0.63). All other habitat types of the study area received a quality index of zero as no successful nest occurred in these habitats. The total area of each type of habitat within each individual pheasant territory, as estimated by the harmonic mean method¹⁸, was then multiplied with the corresponding quality index. Territory quality was finally computed by dividing the sum of quality indices with the total area of the territory. By this method territory quality becomes independent of territory size.

Table 3 Results of the spur length manipulation experiment (December 1987 to June 1988)

Male characteristics	Experimental groups			F	P
	Shortened ($N = 7^*$) $\bar{x}$ (s.d.)	Control ($N = 8^*$) $\bar{x}$ (s.d.)	Elongated ($N = 7^*$) $\bar{x}$ (s.d.)		
Age (yr)	2.1 (1.1)	2.0 (1.1)	1.9 (0.9)	0.14† ($H = 0.23$)	>0.8
Wing length (mm)	214 (5)	213 (4)	215 (7)	0.47	>0.6
Original spur length (mm)	18.3 (3.6)	17.5 (2.5)	17.6 (2.2)	0.20	>0.8
Manipulated spur length (mm)	15.7 (2.1)	18.4 (2.7)	22.1 (3.5)	8.73	0.002
Social rank§	11.7 (9.5)	9.3 (3.9) $N = 7$	11.7 (4.5)	0.33	>0.7
Females per day in April	0.20 (0.40)	0.28 (0.29)	0.87 (0.56)	5.48† ($H = 7.10$)	0.029
Females per day in May	0.17 (0.24) $N = 4$	0.26 (0.27) $N = 7$	0.97 (0.26) $N = 6$	16.1	<0.0005
Females per day in June	0.11 (0.19) $N = 3$	0.09 (0.12) $N = 7$	0.60 (0.47) $N = 6$	4.94‡ ($H = 7.06$)	0.029
Females per day in April–June	0.14 (0.24)	0.26 (0.29)	0.74 (0.43)	6.78	0.006

* Unless otherwise stated in the table. † Residual of dependent variable differed significantly from normality¹⁶. Probability tests refer to Kruskal-Wallis (H). ‡ Cells were significantly heteroscedastic according to Bartlett's test for homogeneity of variances. Probability test refers to Kruskal-Wallis (H). § The most dominant is ranked as 1. || Computed from the average given by the three methods in refs 19, 20, 21, within a distance of 100 m from the male (see Tables 1 and 2). F , One-way ANOVA. At trapping each male pheasant was randomly selected such that the spurs of seven males were clipped at least 2 mm, seven males received elongated, natural coloured and shaped, plastic spurs mounted over the real one so that it was elongated at least 2 mm, and, finally, the spurs of eight males were left intact. After the treatment no male had spurs that were shorter or longer than those measured during 1983–1987 in the natural population. Just before the release from the enclosure the spur length was measured again; reported above as manipulated spur length. In total 22 males and 14 females were included in the study. From the second day after they were released, each pheasant was located by radiotelemetry every weekday throughout June. By the 1 July, ten females, three 'shortened', five 'elongated', and seven 'control' males were still alive. Although no correlations are given in the table it should be noted that the slope of the regression between females per day and manipulated spur length was positive and differed significantly from zero in all months (April, $r = 0.44$; May, $r = 0.66$; June, $r = 0.47$; April–June, $r = 0.51$). An ANCOVA (not shown in the table), controlling for experimental group, reveals that original spur length does not predict females per day in any of the three months ($P > 0.3$ in all cases) (data from April–June: original spur length, $F = 0.43$, $P > 0.5$; experimental group, $F = 6.73$, $P = 0.007$; interaction $F = 0.84$, $P > 0.4$).

Breeding data from 1984–1987, including 45 cases where the females' final mate choice could be determined (defined in Table 2), reveal a significant effect of the mate's spur length on female reproductive success (Fig. 1b). Our judgement of paternity in these 45 cases is confirmed by DNA fingerprint analysis¹³ on 22 day-old chicks from two broods hatched in the study area in 1987, showing that all 22 chicks were sired by the male identified by us as the female's final mate (T. v.S., unpublished data).

We do not know to what extent male genetic quality affects the number of hatched chicks and offspring survival, but we note that the mate's spur length was the sole predictor included in a stepwise multiple regression analysis, with female reproductive success as dependent variable (variables and statistics in Fig. 1). Furthermore, seasonal timing of breeding (date of first egg in the clutch) had no significant influence on female repro-

ductive success ($r = -0.02$, $N = 73$). In fact, the mate, as revealed by his spur length, explains 13% of female reproductive success.

This is the first finding of an intersexually selected male character that correlates not only with male viability⁷, but also with both male and female reproductive success. Our data support recent theoretical work suggesting that viability-based male traits can explain the evolution of mate choice and secondary sexual characters^{1–4}, currently a highly contentious issue^{14,15}.

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Asexual reproduction by oceanic planktotrophic echinoderm larvae

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Cloning among metazoan larvae is rare and generally restricted to a few phyla with parasitic or colonial life-histories. Echinoderms are all non-colonial animals, and although cloning is well documented for some adults, its occurrence in larval stages has not been previously observed¹⁻³. Here we describe a novel mode of cloning by fission in planktotrophic bipinnaria larvae of the sea star *Luidia* sp. from the Sargasso Sea and the Gulf Stream. Reproductive larvae were widely distributed, comprising about 30% of sample populations. This represents an extensive multiplicative potential whereby the pelagic life of the genet is prolonged and the potential of recruitment into benthic adult populations can be enhanced.

Samples were collected in 64 µm-mesh plankton nets towed at discrete depths in the upper 100 m at four stations in the Gulf Stream and the western Sargasso Sea during June and July 1987. Larvae were isolated from other plankters and either maintained in 500 ml pyrex beakers containing filtered sea water and phytoplankton (xenic *Isochrysis galbana*) or fixed immediately in 4% glutaraldehyde buffered with 0.1 M cacodylate at pH 8.1. About 30% ($n=87$) of the bipinnariae collected had highly modified posterolateral arms (Fig. 1a and b), including some arms that were nearly indistinguishable from late gastrula stages of closely related asteroids⁴. Only mid-stage bipinnariae were reproductive. Transformation ultimately resulted in secondary larvae of 300-350 µm in length (Fig. 2), each with an anus, undifferentiated digestive tract and a stomodeum, but no distinct ciliated band. Separation from the primary larvae and subsequent rapid (12-15 h) development into planktonic, early-stage bipinnariae that ingested phytoplankton cells was observed ($n=8$) in ship-board cultures. Primary larvae appear to resume growth and development on completion of the asexual phase; we noted that

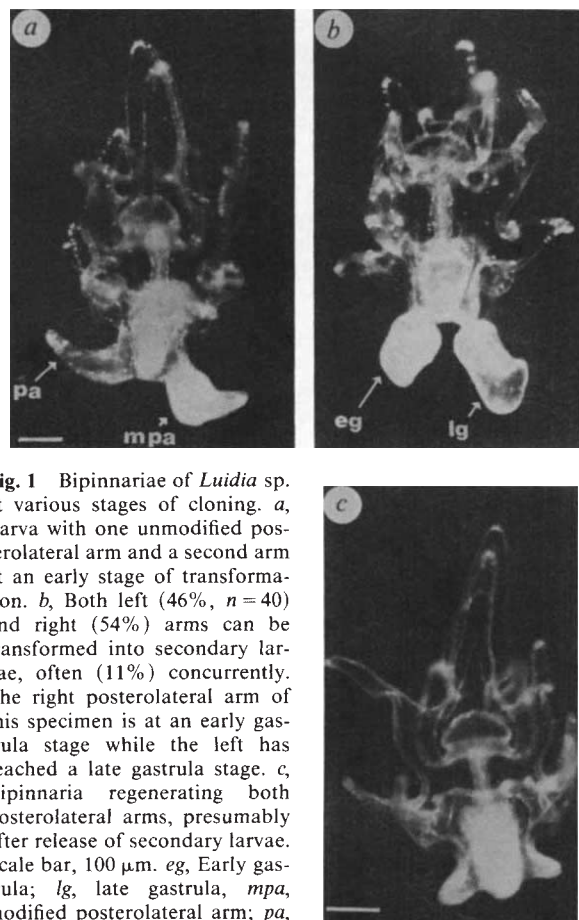


Fig. 1 Bipinnariae of *Luidia* sp. at various stages of cloning. *a*, Larva with one unmodified posterolateral arm and a second arm at an early stage of transformation. *b*, Both left (46%, $n=40$) and right (54%) arms can be transformed into secondary larvae, often (11%) concurrently. The right posterolateral arm of this specimen is at an early gastrula stage while the left has reached a late gastrula stage. *c*, Bipinnaria regenerating both posterolateral arms, presumably after release of secondary larvae. Scale bar, 100 µm. *eg*, Early gastrula; *lg*, late gastrula; *mpa*, modified posterolateral arm; *pa*, posterolateral arm.

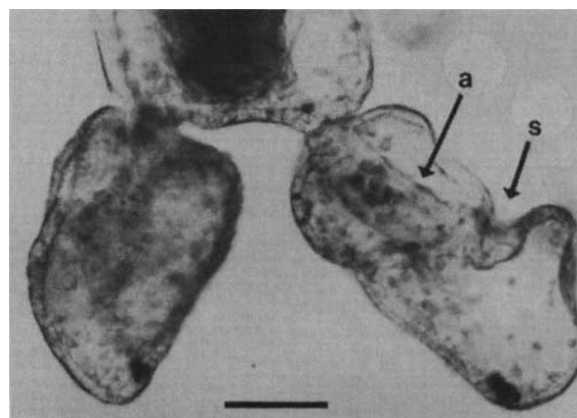


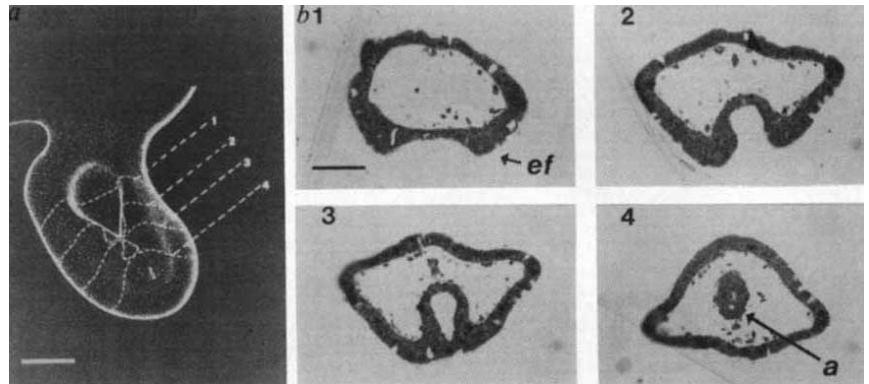
Fig. 2 Close-up of specimen in Fig. 1b. Note the stomodeal pit and archenteron on the late-stage gastrula. Scale bar, 100 µm. *a*, Archenteron; *s*, stomodeal pit.

about 10% of freshly collected, mid-stage bipinnariae were regenerating one or both posterolateral arms (Fig. 1c).

Bipinnariae of the genus *Luidia* are distinctive^{4,5} and can be easily recognized in plankton collections⁶⁻⁸. During early development, their posterolateral arms are typically circular in cross-section; each has a distinct ciliated band that generates both locomotory and feeding currents^{4,5}. To study the transformation from a posterolateral arm to a secondary larva, we examined by light and scanning electron microscopy over 100 bipinnariae at various stages of cloning. Transformation is initiated by expansion of the arm diameter (Fig. 1a), accompanied by proliferation and stratification of the epithelium into multiple cell layers. The densely packed band of cilia along the lateral

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Fig. 3 Gastrulation of a modified postero-lateral arm *a*, Schematic view of the underside of an arm indicating the positions of representative cross-sections in Fig. 3*b*. Invagination is initiated distally and proceeds toward the point of attachment to the primary larva at 90° to the embryonic animal-vegetal axis. Scale bar, 100 μ m. *b*, Photomicrographs of 1 μ m histological cross-sections of an arm embedded in epon resin: (1) first stage of transformation showing a thickened peripheral epithelium with well developed lateral folds; (2) migration of the two lateral folds towards the presumptive mid-dorsal axis of the secondary larva and invagination of tissue formerly occupying that region; (3) fusion of lateral folds, effectively creating an archenteron; (4) blind end of archenteron in a central position along the axis of the arm. Scale bar, 50 μ m. *a*, Archenteron; *ef*, epithelial fold.



margins of the arm dissociates and a uniform pattern of embryonic ciliation emerges. In the meantime, the epithelium joining the arm to the primary larva, destined to be the region of separation between the two, undergoes a progressive constriction. An invagination of the epithelium along the underside of the arm ensues (Fig. 3*a* and *b*) establishing, in an unusual way, a typical gastrular archenteron. The blastopore of the gastrula moves to a central position proximal to the primary larva and remains as the anus. One side of the gastrula, destined to become the oral surface, flattens and imparts a bilobed appearance which is characteristic of bipinnariae⁹. Immediately posterior to the pre-oral lobe, on the flattened side of the secondary larva, a stomodeal invagination breaks through to the undifferentiated digestive tract. At approximately this stage of development the epithelium joining the primary and secondary larvae separates, releasing the secondary larva.

The mode of fission (that is, paratomy) reported here for bipinnariae of *Luidia* sp. is also seen in adult flatworms and annelids¹⁰⁻¹². The process entails extensive morphogenesis of previously differentiated tissue, and culminates in the separation of a recognizable, competent propagule. In the case of *Luidia* sp., after the clone is released the primary larva may enter another clonal phase or resume growth and differentiation leading to metamorphosis. Paratomic fission differs considerably from budding, wherein a new individual originates as an outgrowth of the clonal soma. Proliferation by budding has been implied for some larval echinoderms^{13,14} on the basis of observations that parts of the larval body in several ophiuroids and one asteroid (*Luidia sarsi*) detach from the juvenile primordium at the time of metamorphosis^{13,15}. Cast-off larval bodies of *Luidia sarsi* have been maintained in culture for up to three months, however, and give no indication of production of a second juvenile^{6,15}. Whether budding occurs in post-metamorphic larval ophiuroids remains uncertain.

On the basis of circulation patterns in the North Atlantic¹⁶, we assume that the *Luidia* sp. larvae studied probably originated along the southeastern coast of North America or in the Caribbean. Populations of seven species of *Luidia* that occur in the northwest Atlantic are limited predominantly to relatively shallow (> 450 m) benthic communities¹⁷. Larvae of coastal invertebrates that settle and metamorphose in deep water environments, such as the Sargasso Sea, would probably perish¹⁸. We noted a recruitment rate of some 12% into larval populations of *Luidia* sp. Early (pre-feeding) larval stages of asteroids were not observed in our samples. Therefore, any recruits would be the result of asexual development. The multiplicative potential implied by such recruitment may represent a strategy by which a genet can prolong planktonic development and increase the possibility of at least some ramets being transported to shallow-water regions suitable for settlement and metamorphosis.

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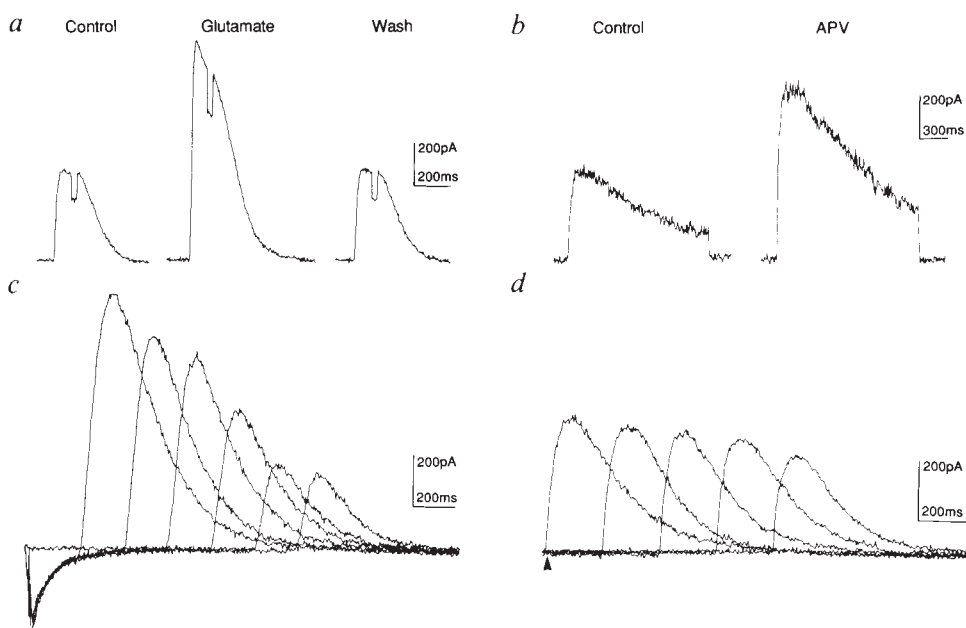
GABA_A responses in hippocampal neurons are potentiated by glutamate

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In the mammalian cortex, glutamate¹ and γ -aminobutyric acid (GABA)² are the principal transmitters mediating excitatory and inhibitory synaptic events. Glutamate activates cation conductances that lead to membrane depolarization whereas GABA controls chloride conductances that produce hyperpolarization. Here we report that the GABA_A-activated conductance in hippocampal pyramidal cells is enhanced by glutamate at concentrations below that required for its excitatory action. The GABA-potentiating effect can be induced, with comparable potency, by several gluta-

Fig. 1 Glutamate enhances GABA-stimulated chloride current. In *a*, 50 μ M glutamate bath-applied, reversibly enhanced GABA chloride current to about 250% compared with respective control currents. GABA (200 μ M, pH 7.4, in Ringer solution) was applied by a 50 ms pressure pulse (25 psi) to a GABA-containing pipette. The neuron was clamped at -10 mV. The recordings in glutamate and wash were performed 1 min after changing respective extracellular saline solutions. Short hyperpolarizing voltage pulses (5 mV) were applied at the peak of GABA-mediated outward current for both GABA and (GABA + glutamate) responses, to estimate conductance changes. Conductance changes caused by glutamate can be estimated by the short hyperpolarizing voltage pulses applied preceding each GABA pulse (data not shown). Bath application of glutamate (50 μ M) produced a sustained inward current (about 15 pA at -10 mV



and about 30 pA at -40 mV) and an increase in resting conductance from 1.91 ± 0.19 nS (mean $\pm$ s.e.m., $N = 34$, at -10 mV) to 2.71 ± 0.25 nS (mean $\pm$ s.e.m., $N = 34$, at -10 mV) (see also Fig. 2a). *b*, GABA (10 μ M) and GABA (10 μ M) + APV (30 μ M) were applied with a multibarrelled inflow system as used for constructing dose-response characteristics (compare with Fig. 3a). For (GABA + APV), both substances were premixed and administered together from a single barrel. The decline in the GABA current during the prolonged application was probably due to desensitization as responses with similar peak amplitudes could be repeatedly activated when GABA was applied at sufficiently long intervals (>45 s). In *c*, glutamate and GABA were applied from different pressure electrodes: glutamate (1 mM, pH 7.4, in Ringer solution) with 20 ms pressure pulses (25 psi), GABA (0.2 mM) with 80 ms pressure pulses (25 psi). GABA pulses were triggered at five different points of time (200 ms up to 1,000 ms, step interval 200 ms) following a glutamate pulse. The GABA response at the far right was triggered without a preceding glutamate pulse and served as a control GABA current. *d*, Recordings using the same experimental protocol as in *c*. The duration of glutamate ejection was adjusted so that the concentration of glutamate was below the threshold for eliciting a glutamate-stimulated inward current: pulse duration 10 ms, pressure strength 15 psi; the delays of GABA pulses following glutamate ejection ranged from 0 to 800 ms in 200 ms steps. The arrow indicates the time of glutamate application, either synchronous to GABA ejection (corresponding GABA current recording at the far left), or preceding the various time-delayed GABA pulses (corresponding recordings of GABA currents at the right). GABA and glutamate concentrations as in *c*. Holding potential in all neurons was -10 mV.

Methods. Slices of the hippocampus from the CA1 region were prepared for trypsin digestion in a bicarbonate-free saline³. Whole-cell clamp recordings¹⁸ were used to monitor the current response to GABA (0.1 to 1 mM in extracellular saline) applied with a picospritzer. The intracellular recording pipette had the following contents (mM): Trizma base, 115; HEPES, 10; leupeptin (a Ca^{2+} -activated neutral protease inhibitor), 0.1; pH 7.3, BAPTA ([1,2-bis(2-aminophenoxy)ethane - N , N , N' , N' -tetra-acetic acid]), 10 (BAPTA has the same affinity for Ca^{2+} as EGTA but binds it faster¹⁷); MgCl_2 , 4; ATP-Na_2 , 2. The pH was adjusted to 7.3 by adding Tris(methanesulphonate). NaCl was added (12 mM) to nullify the junction potential at the Ag/AgCl-recording solution interface. BAPTA, Mg^{2+} and ATP serve to stabilize GABA currents¹¹. Tightly controlled $[\text{Ca}^{2+}]_i$ (by inclusion of 10 mM BAPTA into the recording pipette) was required to avoid fluctuations in $[\text{Ca}^{2+}]_i$, which may affect GABA chloride currents¹¹. The external solution consisted of (mM): NaCl, 140; KCl, 5; CaCl_2 , 2; MgCl_2 , 1; HEPES, 10; D-glucose, 25; pH, 7.4. External perfusion was applied at a rate of 5 ml min⁻¹, close to the cell to prevent agonist-induced desensitization. All GABA and glutamate solutions were made up in Ringer solution, the pH adjusted to 7.4. Experiments were performed at 18–22 °C. GABA responses were elicited by pressure pulses (10 to 80 ms) at a low frequency (0.033 Hz) to avoid cumulative desensitization. Step hyperpolarizations (-10 mV, 10 ms) were applied before eliciting GABA responses, to monitor the leak conductance and input capacitance. The average value for input resistance was 522 ± 32 M Ω and membrane capacitance was 14.6 ± 3.4 pF (mean $\pm$ s.e.m., $N = 34$, -10 mV holding potential). The GABA current was completely blocked by 10 μ M picrotoxin. This fact, together with a reversal potential at the chloride equilibrium potential, suggests that the GABA_A receptor is activated¹⁹.

mate analogues such as quisqualate, *N*-methyl-D-aspartate (NMDA), kainate and, surprisingly, by D-2-amino-5-phosphonovalerate (APV), an antagonist for NMDA receptors. Data from dose-response curves show that glutamate enhances the GABA_A conductance without significantly changing GABA binding affinity. The low concentration of glutamate needed to enhance GABA_A responses raises the possibility that glutamate modulates the strength of GABA-mediated transmission in the cortex.

Acutely dissociated cells from the CA1 hippocampal subfield of adult guinea pigs³ were used to examine the effects of glutamate on neuronal GABA responses. Figure 1a shows current responses of an isolated CA1 pyramidal cell to GABA (200 μ M) applied by pressure ejection pulses of 50 ms. The cell was clamped at -10 mV. Bath application of L-glutamate (50 μ M) resulted in a reversible increase of GABA-induced outward current. The potentiation of GABA_A conductance by glutamate was observed in all 34 cells tested at this (50 μ M) glutamate concentration.

Glutamate applied at similar concentrations also activates inward currents⁴. To compare the time course of this effect with its potentiating action on the GABA response, short pulses of GABA and glutamate were applied by ejection from two different pressure pipettes. Glutamate pulses, applied by pressure ejection, were followed at varying intervals by a fixed pulse of GABA (Fig. 1c, d). GABA responses elicited within one second after glutamate application were potentiated. The potentiation effect was largest at the shortest GABA-pulse delay (in this cell about 200 ms) and monotonically decreased with increasing intervals between glutamate and GABA application. This effect was observed in all 10 cells tested, although there was some variability between cells in the amount of GABA-current enhancement. The effect of glutamate on the GABA current significantly outlasted the time course of the glutamate-mediated inward current, suggesting that the enhancing action may have a higher sensitivity to glutamate. When the amount of applied glutamate was reduced by shortening the duration

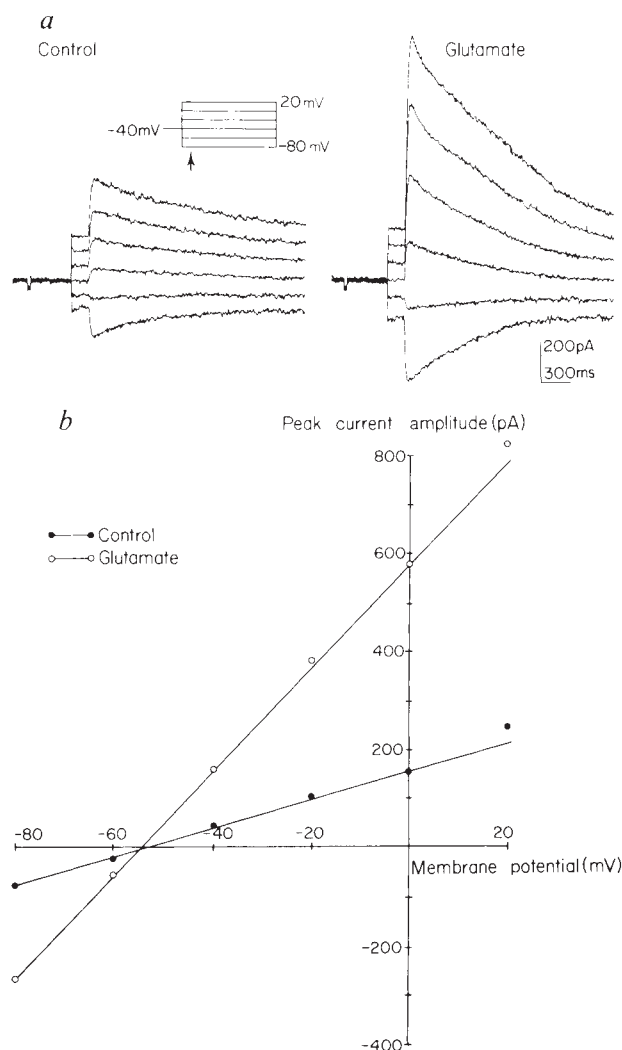


Fig. 2 Effect of bath-applied glutamate on GABA-stimulated currents at various membrane potentials. *a*, Left, control responses to pressure-ejected GABA (70 ms, 25 psi) from GABA-containing pipette (0.2 mM) for six different membrane potentials from -80 to +20 mV in steps of 20 mV; right, the same protocol in a solution containing 50 μ M glutamate. The inset on the left illustrates the experimental protocol, the arrow indicating the time point of GABA ejection. *b*, Graph of peak current amplitude of GABA-stimulated currents versus membrane potential in control and glutamate-containing solutions. Reversal potentials for both currents (GABA, and GABA+ glutamate) were estimated from straight lines drawn between the values of -80 and 0 mV. Similar results were obtained for 14 other cells using this experimental protocol. Outward rectification of GABA current was observed in seven out of 14 cells tested under these conditions but was not observed in the cell depicted here. Both linear and non-linear GABA IV relationships have been described²⁰⁻²². Glutamate enhancement was observed in both types of cells.

of the pressure pulse such that the concentration reaching the cell did not elicit any detectable inward current, enhancement of GABA current was still observed at short intervals following the glutamate pulse (Fig. 1*d*). In this example GABA enhancement was maximal when GABA and glutamate were simultaneously applied.

Potential of the GABA response by glutamate is not accompanied by a shift in the reversal potential for the GABA response. Measurements at various holding potentials revealed a proportional increase at a fixed glutamate concentration. Figure 2 shows control GABA responses and GABA responses in 50 μ M glutamate. The current-voltage relation in Fig. 2*b* depicts the proportionate enhancement by glutamate at holding potentials

between -80 and +20 mV (Fig. 2). These data indicate that glutamate causes a change in the slope conductance of the GABA *I*-*V* relation.

In another series of experiments the effect of several glutamate analogues on GABA currents was examined. Quisqualate, kainate and NMDA potentiated GABA_A responses. The potentiating effect was not blocked by several conventional antagonists of glutamate receptor subtypes, including APV and γ -D-glutamylglycine (γ -DGG). Surprisingly, the NMDA-receptor antagonist APV mimicked the effects of glutamate on GABA chloride currents (Fig. 1*b*). Sequential application of 50 μ M glutamate, quisqualate, NMDA and APV produced 235%, 485%, 341% and 215% enhancement of GABA current in another cell. On average, at concentrations of 50 μ M, glutamate potentiated GABA responses to $252 \pm 19\%$ (mean $\pm$ s.e.m., $N = 34$), quisqualate $439 \pm 23\%$ ($N = 6$), kainate $184 \pm 7\%$ ($N = 4$), NMDA $318 \pm 29\%$ ($N = 9$) and APV $173 \pm 16\%$ ($N = 5$) where N is the number of neurons tested. In all these experiments neurons were clamped at -10 mV.

To examine the mode of action of glutamate on GABA currents, GABA dose-response curves were constructed in the absence and presence of glutamate (Fig. 3*a*). Various GABA concentrations were applied by a multi-barrelled inflow system close to a given cell. Application of GABA together with 50 μ M glutamate produced a marked increase in the maximum peak of the GABA current. Neither the threshold for GABA nor the GABA concentration for half-maximal response were significantly altered, indicating that glutamate does not affect GABA binding affinity (Fig. 3*a*). The potentiating effects of glutamate, at different concentrations, on GABA responses is shown in Fig. 3*b*. GABA (200 μ M in a pipette) was applied by pressure ejection (50 ms), glutamate by bath perfusion. The potentiating effect became significant at a glutamate concentration of 10^{-8} M (108.8 ± 2.0 , $N = 14$, compared with control of 100%, $P < 0.01$, *t*-test), the binding affinity constant (K_D) of the curve was 10.2 μ M. These values are significantly lower compared with those reported for glutamate-activated inward currents (10 μ M for threshold, 1.1 mM for K_D (ref. 4) and NMDA-mediated inward currents (> 1 μ M for threshold⁵).

The observed facilitation of GABA by glutamate might result from an alteration of the GABA receptor complex as a result of the enzymatic treatment in the dissociation procedure. Consequently we examined the glutamate action in hippocampal cells isolated without enzymatic treatment by omitting trypsin from the dissociation procedure. The yield of isolated neurons following this procedure was significantly reduced. Successful recordings from three neurons isolated in this way show that in all cases glutamate potentiated the GABA responses. Using the experimental protocol shown in Fig. 1*a*, 50 μ M glutamate in the perfusate reversibly enhanced the GABA chloride current to about 250% of control.

There may be two ways in which excitatory amino acids modify GABA_A receptor conductance. First, the glutamate analogues may activate their corresponding receptor subtypes and trigger a common, probably intracellular process leading to the modification of the GABA response. Alternatively, the various agents may act at a common binding site at, or closely associated with, the GABA_A receptor complex to elicit this novel action. In either case the process underlying the GABA_A conductance increase can be considered to be distinct from that leading to the opening of cationic conductances as the two responses differ considerably in their concentration requirement and in their selectivity towards agonists.

Previous studies show that a number of pharmacological and possibly endogenous agents can bind to the GABA_A receptor and affect its efficacy (for review see refs 6-9). Benzodiazepines and barbiturates, although acting at different sites, potentiate GABA responses by changing the binding affinity of the GABA recognition site by an allosteric modification. The glutamate action is probably distinct from the mechanism of ben-

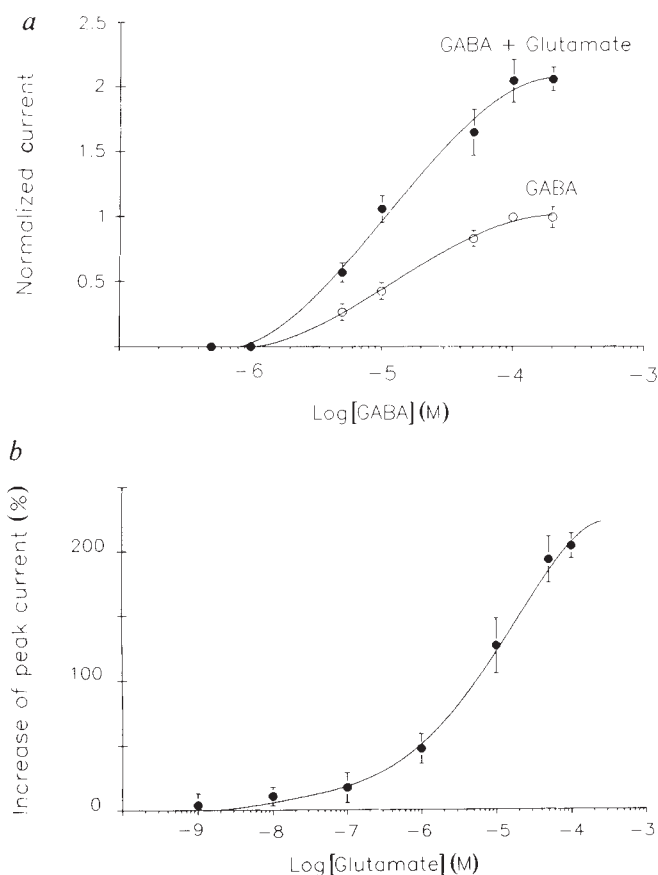


Fig. 3 Dose-response curves for GABA and (GABA + glutamate). **a**, GABA responses were elicited by direct application of various concentrations of GABA-containing solutions to the cell. Rapid solution changes were effected using a multibarrelled inflow system²³. Time to peak of the GABA responses was within 100 ms with this approach. Glutamate (50 μ M) was administered together with GABA from the same barrel. All GABA and (GABA + glutamate) solutions were made up in Ringer (pH 7.4). GABA responses are normalized averages of 4–11 values (responses at 100 μ M 'GABA' = 1). The average K_D ($\pm$ s.e.m.) for GABA obtained from dose response curves of eight cells was 12.40 ± 1.64 μ M, and for (GABA + glutamate) 10.86 ± 1.49 μ M ($N=9$); n , where n = number of binding sites, was 1.18 ± 0.14 for GABA and 1.25 ± 0.21 for (GABA + glutamate). Neither K_D nor n for GABA and (GABA + glutamate) was significantly different ($P > 0.1$, t -test). **b**, Increase of GABA current as a function of glutamate concentration. GABA was applied by pressure ejection at a fixed concentration (200 μ M), and the various glutamate concentrations by bath perfusion. Data are represented as percentage increase of peak current (mean $\pm$ s.e.m., $N=5-14$) compared with GABA responses in control Ringer. ($K_D = 10.2$ μ M and $n=0.61$).

Methods. Dose-response curves were fitted using a non-linear least-square fitting program to the equation $I = I_{\max} \times ([A]/([A] + K_D))^n$ with $[A]$ the dose of GABA in presence or absence of glutamate. $I_{\max}$ (the maximum response), n and K_D were allowed to float to obtain the best fit.

zodiazepines and barbiturates, as glutamate does not change the binding affinity for GABA. It is possible that glutamate modifies kinetic properties of single GABA channels or transforms non-functional receptor-channel complexes into functional ones.

The potentiating effect of glutamate on the GABA response was examined under conditions where fluctuation of $[Ca^{2+}]_i$ is minimized. Without such experimental manipulation, glutamate may suppress GABA sensitivity by elevating $[Ca^{2+}]_i$ ^{10–12}. In this regard, the high sensitivity of glutamate in potentiating GABA responses may be significant in that it could allow the isolated expression of this effect without activation of cationic (par-

ticularly Ca^{2+}) conductances (see Fig. 1c). In addition, the low concentration of effective glutamate for the potentiating effect, compared with the level of glutamate reported to exist in the cerebral spinal fluid (1–10 μ M)^{13,14} raises the possibility that glutamate may affect the strength of synaptic inhibition *in vivo*. Interestingly, similar modulations of excitatory transmission by endogenous substances have been suggested on the basis of the recent discovery that low levels of glycine potentiated NMDA responses¹⁵. Finally, regulation of inhibition by synaptically released glutamate *in situ* is also plausible as recent results show that GABA and glutamate were co-released from GABAergic synaptosomes of cerebral cortex¹⁶.

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Differential activation of myotube nuclei following exposure to an acetylcholine receptor-inducing factor

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A glycoprotein purified from chick brain, of relative molecular mass 42,000, increases the rate of appearance of acetylcholine receptors (AChRs) on the surface of chick myotubes¹. RNase protection assays have shown that this AChR-inducing activity (ARIA) increases the amount of mRNA encoding the α -subunit of the AChR, with little or no effect on the amounts of γ - and δ -mRNAs². Here, we report that the mRNAs encoding the α - and γ -subunits of the receptor detected by *in situ* hybridization are concentrated around nuclei in cultured myotubes. Consistent with previous results, ARIA selectively increased the amount of α -subunit mRNA, but we now find that all nuclei were not activated to the same extent, with a substantial number not responding at all. Assuming that ARIA is released by motor nerve terminals, our results indicate that only a subset of muscle nuclei are capable of contributing to the accumulation of AChRs at developing neuromuscular junctions.

Fig. 1 ARIA increases the amount of α -subunit mRNA detected by *in situ* hybridization. Phase contrast (*a,c,e*) and dark-field (*b,d,f*) micrographs of control (*a-d*) and ARIA-treated (*e,f*) myotube cultures. *a, b, e, f* were hybridized with an 35 S-labelled anti-sense probe for AChR α -subunit mRNA. *c* and *d*, Hybridization with a probe spanning the same region of the α -mRNA, but in the sense orientation. Note that myotubes are not labelled uniformly by the anti-sense probe; areas containing clusters of nuclei display a high density of silver grains. Fibroblasts are not labelled above background. Scale bar, 100 μ m.

Methods. Mononucleated cells dissociated from 11-day-old embryonic chick pectoral muscle were plated on collagen-coated 12-mm glass coverslips at a cell density of 9×10^4 per cm^2 (ref. 10). Some cultures were treated for 24 h beginning on the third day after plating with a preparation of ARIA of relative molecular mass 42,000 partially purified by high pressure liquid chromatography from an acid extract of chicken brains¹. On the fourth day after plating cells were fixed in 4% paraformaldehyde and 70% ethanol. Myotubes were prehybridized for 1–3 h at 43 °C in 50% formamide, 5 $\times$ SSC, 0.3 M dithiothreitol, 1 $\times$ Denhardt's, and 500 $\mu\text{g ml}^{-1}$ salmon sperm DNA, and were then hybridized overnight at 43 °C in the same solution containing 200 ng ml^{-1} of 35 S-labelled RNA probe (2×10^9 d.p.m. μg^{-1}). The 600-nucleotide RNA probes were transcribed in the sense or anti-sense direction from the region between a *Pvu*II site and an *Eco*RI site at the 3' end of a chick α -subunit cDNA. The anti-sense probe was synthesized from the T7 promoter of pGEM-1 after linearization with *Pvu*II (see Fig. 1D of ref. 2). The sense probe was synthesized from the SP6 promoter of pGEM-3Z after linearization with *Eco*RI; this template contains the *Eco*RI–*Pvu*II fragment of the α -subunit cDNA. Coverslips were washed in succession with: 4 $\times$ SSC and 0.3 M β -mercaptoethanol (1 h at 43 °C); 20 $\mu\text{g ml}^{-1}$ RNase A, 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol (0.5 h at 37 °C); 2 $\times$ SSC and 0.3 M β -mercaptoethanol (0.5 h at 43 °C); 0.1 $\times$ SSC and 0.3 M β -mercaptoethanol (1 h at 43 °C). In some experiments the final wash was at 65 °C, or an additional wash was performed in 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8), 1 mM EDTA, 10 mM dithiothreitol (10 min at 65 °C). These modifications reduce the background level of hybridization. Coverslips were mounted on glass microscope slides, dipped in NTB2 emulsion, and exposed at 4 °C for 10–25 days. Slides were developed for 2.5 min in Kodak D-19, rinsed in water, and then fixed for 5 min in Kodak fixer, all at 15 °C.

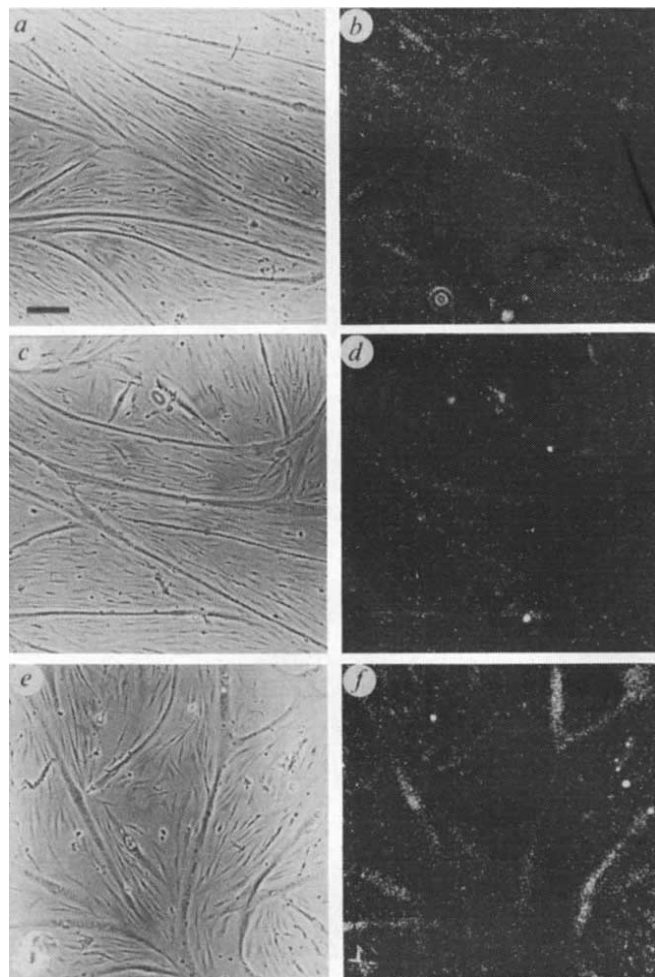
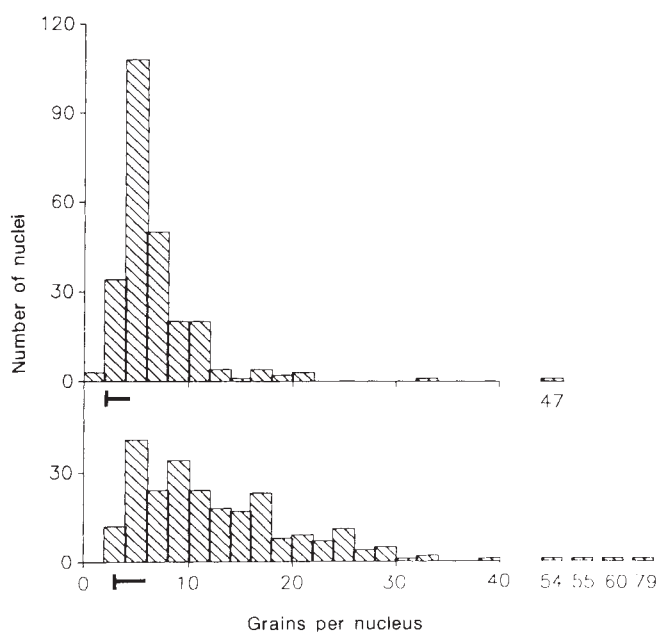


Fig. 3 Histograms of perinuclear grain counts over control and ARIA-treated myotubes hybridized with the α -mRNA probe. Top, control; bottom, ARIA-treated. The small bars below each abscissa indicate the mean grain count per nucleus plus two standard deviations for myotubes hybridized with the sense-strand probe. **Methods.** Myotubes were treated with ARIA for 24 h beginning on day 6 after plating, and were fixed on day 7 and hybridized with the α -subunit probe (see Fig. 1 legend). Using a microscope drawing tube a 30 μm circle was positioned over 100 randomly chosen areas containing 1–5 myotube nuclei visualized with bisbenzimidazole, and the number of silver grains within the circle was counted under dark-field illumination. The mean number of grains per nucleus was then calculated for each circle. Circles contained an average of 2.5 nuclei; 15 circles (control cultures) and 21 circles (ARIA-treated cultures) contained a single nucleus. The median values for each histogram are 5.3 grains/nucleus (control, $n = 251$), and 10.7 grains/nucleus (ARIA-treated, $n = 245$). Background hybridization was quantitated by counting the number of grains produced by the sense-strand probe in 20 circles; each count was divided by 2.5, the average number of nuclei per circle, before calculating the mean and standard deviation.



Untreated chick myotubes hybridized *in situ* with an 35 S-labelled RNA probe complementary to α -subunit mRNA (Fig. 1*a,b*) were labelled above the background levels observed over fibroblasts in the same cultures or over myotubes in parallel cultures hybridized with a sense-strand probe (Fig. 1*c,d*). Overnight treatment of myotubes with a partially purified preparation

of ARIA dramatically increased the amount of α -mRNA (Fig. 1*e,f*). Purified ARIA material representing overall purifications of 500- to 40,000-fold all had a similar effect.

Close examination of Fig. 1 shows that the distribution of α -mRNA is not uniform within individual myotubes. The density of grains is particularly high around nuclei (see Figs 2*a*

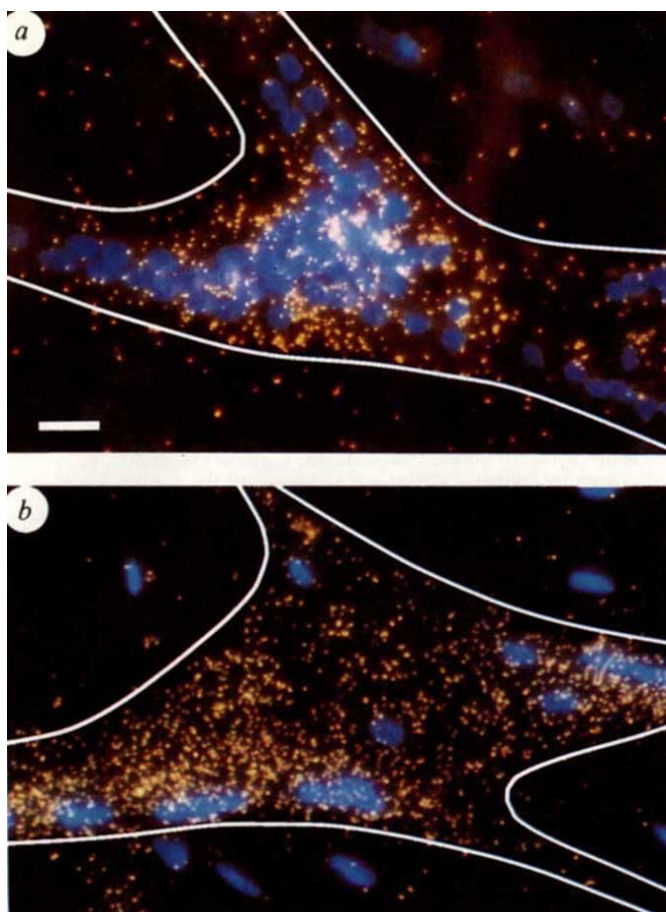


Fig. 2 α -subunit mRNA is concentrated around myotube nuclei, whereas actin mRNA is not. These dark-field micrographs were taken under ultraviolet illumination to show bisbenzimidazole-stained nuclei (bisbenzimidazole is a fluorescent dye, Hoechst 33342). Myotubes were hybridized with a probe for AChR α -mRNA (*a*), or with a probe specific for chick α -cardiac actin (*b*). The outlines of each cell (observed under phase-contrast illumination) are indicated by the white lines. α -cardiac actin mRNA is abundantly expressed in chick skeletal myotubes¹¹. The actin probe was transcribed from a *Bgl*III–*Ava*II fragment of the 3' untranslated region of a chick α -cardiac actin cDNA¹². This probe does not hybridize to mRNAs encoding other actin isoforms. Scale bar, 15 μ m.

and 4a). Silver grains are most concentrated at the periphery of nuclei, a distribution that probably reflects the location of rough endoplasmic reticulum where receptor subunit mRNAs are translated³. In contrast, when cells were hybridized with an actin cRNA probe, silver grains were distributed more uniformly throughout the cytoplasm (Fig. 2b). The same result was obtained when myotubes were hybridized with [³H] poly(U) (data not shown), which should detect all poly(A)⁺ mRNAs.

As expected from our solution hybridization studies², the level of γ -subunit mRNA detected by *in situ* hybridization in untreated myotubes was greater than that of α -mRNA, and it was not further increased by ARIA. γ -mRNA was also concentrated around nuclei (data not shown).

The relation between α -mRNA and myotube nuclei was quantitated by counting the silver grains within a circle of 30 μ m in diameter that was positioned over nuclei before visualizing the grains under dark-field illumination. Myotube nuclei tend to cluster, so when more than one nucleus was present within the circle the average number of grains per nucleus was calculated. In control cultures, more than 90% of the 251 nuclei analysed displayed an average grain count of less than 12 (Fig. 3). The histogram was skewed, however, with a few nuclei

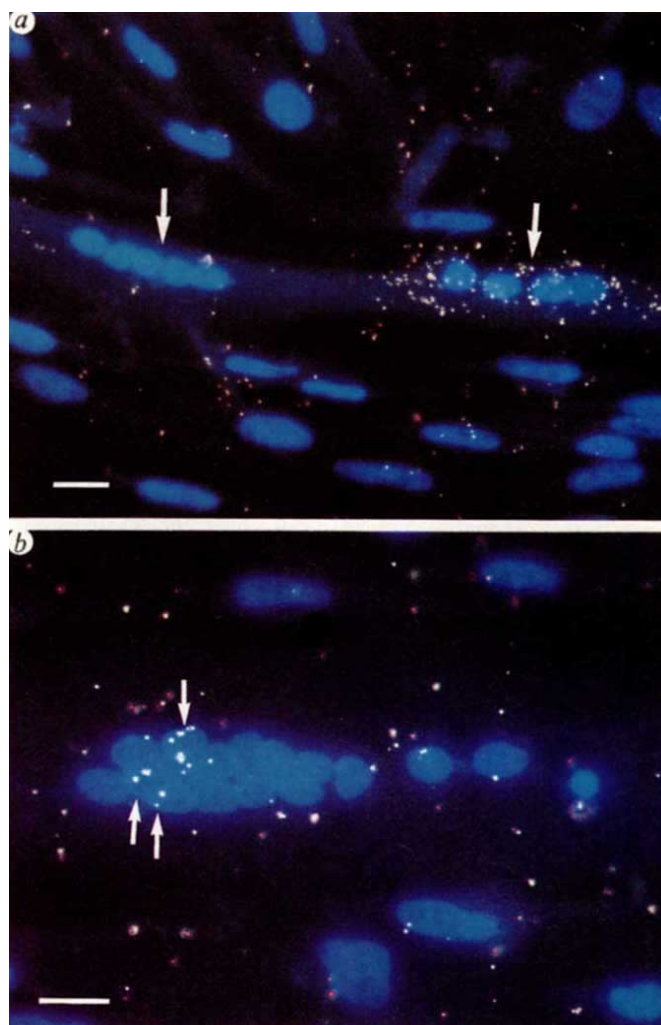


Fig. 4 Nuclei within a single ARIA-treated myotube express different amounts of α -subunit mRNA. The micrograph in *a* shows two adjacent groups of nuclei (arrows) in a culture hybridized with the α -mRNA probe. The right hand group is heavily labelled with silver grains, whereas the left hand group is not labelled above background. The micrograph in *b* shows a group of myotube nuclei in a culture hybridized with an ³⁵S-labelled RNA probe complementary to 70 nucleotides of intron 6 of the α -subunit gene². (This probe was transcribed from the SP6 promoter of *Xba*I-linearized pGEM-1 containing a blunt-ended *Hind*III–*Dde*I fragment¹³ of the α -subunit gene.) Only a few of these nuclei are labelled (arrows); silver grains are concentrated directly over them, rather than at their periphery as is the case for the exon-containing α -subunit probe (compare with *a*). Scale bars, 15 μ m.

labelled much more intensely. 22% of the nuclei were not labelled above background (less than two standard deviations above the mean grain count produced by the sense-strand probe).

The distribution in ARIA-treated cultures was markedly different (Fig. 3). 45% of the 245 nuclei displayed an average grain count greater than 12. As the largest values in both treated and control cultures were similar, ARIA may trigger cellular events that occur at low frequency in untreated myotubes (see discussion in ref. 4). It is significant that the majority of nuclei in ARIA-treated cultures were not labelled more heavily than the main peak in control cultures. As the percentage of nuclei in ARIA-treated cultures that were labelled at background levels (26%) is comparable to control cultures (22%), the simplest interpretation is that a subpopulation of myotube nuclei do not respond at all to the inducing factor.

Significant differences in the amount of perinuclear α -mRNA were evident even in a single ARIA-treated myotube. In Fig. 4a, one cluster of nuclei is heavily labelled whereas another group, approximately 50 μ m away in the same cell, is not labelled above background. Previous studies have shown that high density clusters of surface AChRs occur near myotube nuclei⁵⁻⁷. We have noted a correlation between the local concentration of α -mRNA and the overall density of surface AChRs in the same region, but the precise spatial relationship between these molecules remains to be determined.

Marked differences among nuclei in ARIA-treated cultures were also evident after hybridization with a probe that detects a partially spliced nuclear RNA encoding intron 6 of the α -subunit gene² (Fig. 4b). The striking heterogeneity in the distribution of the exon probe is therefore probably due to different rates of transcription of the α -subunit gene, and cannot be attributed entirely to extra-nuclear factors such as the distribution of rough endoplasmic reticulum. We have not yet reliably detected a signal from the intron probe in control cultures, probably because the level of intron 6-containing nuclear RNA is only 16% of that of mature α -mRNA².

Our results indicate that ARIA does not activate all myotube nuclei to the same degree. The distribution of grain densities in ARIA-treated cultures was not obviously bimodal, but we averaged grain counts over more than one nucleus in many measurements, minimizing the distinction between responding and non-responding nuclei. Likewise, the resolution of our method does not allow us to determine if the distribution of responding nuclei is graded or multimodal. In addition, we do not know if the nuclei labelled at background levels are a distinct population, or part of the same distribution as nuclei labelled above background. These issues might be resolved if the signal-to-noise ratio produced by the intron probe can be improved.

We cannot at present account for the observed differences among nuclei. No characteristic morphological features of nuclei were noted that correlate with their level of expression of α -mRNA. The ability to respond to inducing factors may be an intrinsic property of nuclei derived from a particular myogenic lineage. Alternatively, the ability to respond may be determined by the cytoplasmic environment of the nuclei. It is unlikely that the heterogeneity is related to the distribution of receptors for ARIA, because we have observed similar differences among nuclei in α -mRNA expression following treatment with tetrodotoxin (unpublished data). It may also be relevant in this regard that only a few nuclei in denervated chick muscle (15-days after hatching) seem to be heavily labelled with an α -subunit mRNA probe⁸.

Northern blot and *in situ* hybridization experiments have shown that AChR subunit mRNAs are concentrated at synaptic sites *in vivo* (refs 8, 9, and unpublished results). Although it is clear that motor neurons induce the accumulation of receptors in the postsynaptic membrane, our results indicate that myotube nuclei are not equally susceptible to this trophic influence. A subset of nuclei might be predisposed to participate in synapse formation even before the nerve arrives.

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Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts

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An important corollary to the recent advances in our understanding of the primary cause of Duchenne muscular dystrophy¹⁻⁶, is the validation of genuine genetic homologues as animal models of the disease^{7,8} in which potential therapies can be tested. The persistent skeletal muscle necrosis that characterizes human Duchenne muscular dystrophy⁹ is also seen in the mdx mouse¹⁰⁻¹³ and is, in both, a consequence of a deficiency of dystrophin^{6,7}, probably within the muscle fibres themselves¹⁴⁻¹⁶. As injected muscle precursor cells of one genotype can fuse with host muscle fibres of a different genotype and express the donor genes^{17,18}, we decided to test grafts of normal muscle precursor cells to see if they could induce synthesis of dystrophin in innately dystrophin-deficient mdx muscle fibres. We show that injected normal muscle precursor cells can fuse with pre-existing or regenerating mdx muscle fibres to render many of these fibres dystrophin-positive and so to partially or wholly rescue them from their biochemical defect.

Mononucleated cells were prepared by enzymatic dissociation of normal neonatal mouse muscle¹⁸. Between 5×10^5 and 4×10^6 cells were injected into the right extensor digitorum longus (EDL) muscles of 5-27-day-old mdx hosts. Mice were killed after 20-99 days and the EDL and adjacent tibialis anterior and peroneus muscles removed from the injected and contralateral non-injected legs. Successful fusion of donor muscle precursor cells (mpc) into host (mdx) myofibres was verified by isoelectric focusing of allotypic isoenzymes of glucose 6-phosphate isomerase (GPI EC 53-1-9)¹⁷ (Fig. 1). Donor mpc, homozygous for *GPI-1s^b* (producing the BB isoenzyme) were injected into homozygous *GPI-1s^a* hosts (producing the AA isoenzyme). The AB GPI heterodimer can be formed only in mosaic host/donor myofibres in which both types of myonucleus express their individual *GPI-1* alleles. 'Host' mdx strains (designated mdx/nude and M9) were bred to be homozygous for the *GPI-1s^a* allele and also to avoid immune rejection of the injected cells. Athymic 'mdx/nude' double mutants accept a wide range of allografts, whereas the M9 strain is MHC-compatible with the donor strains and is readily made tolerant to these strains at birth¹⁹.

Of 70 mice analysed, 39 had the heterodimer AB GPI in one or more muscles, indicating fusion of the injected cells into host (mdx) myofibres (Table 1; Fig. 1). In 18 muscles the proportion of AB heterodimer exceeded that of the donor (BB) isoenzyme (see for example Fig. 1b, c and e), indicating that the majority of surviving donor nuclei were resident in mosaic muscle fibres. Grafts of normal mpc succeeded less frequently in young (5-7 day) mouse muscles (3 of 17), in which myofibres should be acquiring new myonuclei during rapid growth²⁰, than in regenerating muscle of 19-27-day-old mice (36 of 53). In the older

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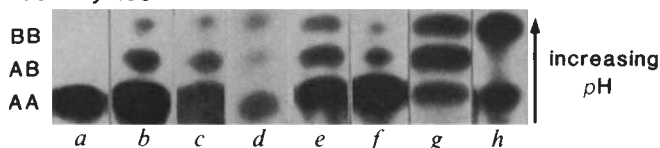
GPI
isoenzymes

Fig. 1 GPI isoenzyme analysis of mdx muscles injected with mpc derived from normal (C57Bl/10+/+) donors (lanes a–e) or mpc derived from mdx (C57Bl/10 mdx/mdx) donors (lane f). a, EDL muscle of mdx/nude host, injected with 1.5×10^6 normal mpc at 7 days and examined 42 days later. This muscle contained only the host AA GPI isoenzyme; presumably the injection had failed. b, EDL muscle of M9 host, injected with 1.5×10^6 normal mpc at 21 days and examined 50 days later. Host AA, donor BB and the AB heterodimer isoenzymes were present. c, TA muscle of mdx/nude host, adjacent to an EDL which had been injected with 5×10^5 normal mpc at 23 days and examined 40 days later. Host AA, donor BB and the AB heterodimer isoenzymes were present. d, EDL muscle of mdx/nude host, injected with 1.7×10^6 normal mpc at 25 days of age and examined 50 days later. Host AA, donor BB and the AB heterodimer isoenzymes were present. e, EDL muscle of mdx/nude host, injected with 1×10^6 normal mpc at 27 days and examined 20 days later. Host AA, donor BB and the AB heterodimer isoenzymes were present. f, EDL muscle of mdx/nude host, injected with 5×10^5 mdx mpc at 24 days and examined 21 days later. Host AA, donor BB and the AB heterodimer isoenzymes were present. This indicates that the donor mdx cells had become incorporated into the host mdx muscle fibres as myonuclei. No dystrophin was detected in such muscles. g, Standard: skeletal muscle from an F₁ (C57Bl/6J $\times$ 129/ReJ) mouse, to show the location of the AA, BB and heterodimer AB GPI isoenzymes. h, Control: a mixture of C57Bl/6J and 129/ReJ skeletal muscles, to show that the AA and BB GPI isoenzymes do not reassociate to form the heterodimer AB GPI isoenzyme during isoelectric focusing.

Methods. Glucose-6-phosphate isomerase isoenzymes were separated by isoelectric focusing on 0.8 mm agarose gels of extracts from individual 5 μ m cryostat sections. The enzyme was visualized with an agarose overlay containing fructose-6-phosphate, glucose-6-phosphate dehydrogenase, Nitroblue Tetrazolium and co-factors²³.

mice, fusion of donor mpc into host muscles was significantly more frequent in the nude/mdx (27 of 34) hosts than in M9 hosts (9 of 19) ($P = 0.038$; 2×2 , Yates-corrected χ^2 test), suggesting that there was some immunological impediment to mpc grafting, even in hosts that are MHC-compatible and tolerant. Heterodimeric GPI was sometimes found in a muscle adjacent to the injected EDL (see for example Fig. 1c) where it may indicate inaccurate injection or migration of mpc between contiguous muscles^{21,22}. Heterodimeric GPI was never observed in the non-injected muscles of the contralateral leg.

To see if the normal mpc that had been incorporated into mdx myofibres could produce dystrophin, selected muscles containing a high proportion of heterodimeric GPI were tested for their dystrophin content by immunoblotting and by immunofluorescent staining. The immunoblot in Fig. 2 of injected mdx EDLs from two mice indicates that dystrophin of normal size is present at up to 30–40% of normal levels, as judged by densitometry. No dystrophin was detected in the non-injected contralateral EDL. Immunofluorescent staining of injected muscles showed that dystrophin was in its normal sarcolemmal location^{23–25} (Fig. 3) in some 10–40% of muscle fibre profiles. In some muscles, dystrophin-positive fibres were distributed diffusely (Fig. 3a); in others they formed foci. In addition, individual fibre profiles often showed both dystrophin-positive and dystrophin-negative regions; this patchy pattern is strikingly similar to that observed in young (10-day-old) mdx/+ heterozygotes²⁶, suggesting a similar type of local expression of dystrophin from competent, admixed with non-competent,

Table 1 Summary of implantation experiments and results of GPI analyses

Host	Donor	Host age (days)	Incidence of heterodimer* GPI in host	
			EDL	Adjacent muscle
mdx/nude	Normal†	5–7	1/9	1/9
mdx/nude	Normal†	19–27	23/34	14/34
M9	Normal†	5	0/8	2/8
M9	Normal†	21–22	6/19	5/19
mdx/nude	mdx‡	24	8/12	2/12

Two mouse colonies were generated to act as hosts. The mdx mutation was bred from its normal GPI-1s^b strain (C57Bl/10) on to a background homozygous for the *GPI-1s^a* allele as it is easier to detect small amounts of donor cells of *GPI-1s^b* allotype against this background than *vice versa*. At the same time, to avoid allograft rejection, we arranged for the host mouse to be either of the same H-2 type as the donor of the mpc, or a nu/nu mouse in which the defective immune system permits allograft survival. To achieve the former, mdx female mice were initially crossed with male 129/ReJ (GPI-1s^a) mice (both strains H-2^b). The resultant F1 progeny were self-bred and breeding stock was selected from the F2 generation. F2 mice that were *GPI-1a^a* allotype and, as assessed by muscle biopsy, mdx, were crossed to obtain a colony (designated M9) of pure-bred mdx mice homozygous for *GPI-1s^a* and H-2^b. Tolerance was induced in prospective M9 hosts by injection at birth of 5×10^6 donor strain spleen cells into the orbital branch of the anterior facial vein. A similar breeding strategy was used to obtain a colony of nu/nu mdx mice from female mdx mice crossed with male nu/nu mice. Here in the F2 generation nu/nu mdx male mice were mated with non-nude female mdx mice (nu/nu mice are poor mothers). Of the latter, only those identified as nu/+ (whose litters included nu/nu progeny), were retained as a basis for the breeding colony. Here too we selected mice homozygous for *GPI-1s^a*. Into the muscles of these mdx/nude mice or tolerant M9 mice, we injected normal mpc, prepared from newborn C57Bl/10 mice as previously described¹⁷. Mpc were implanted by percutaneous injection into growing muscles of 5–7 day old mice as previously described¹⁸. Mice 19–27 days old were anaesthetized, a slit was made in the skin overlying the EDL and the mpc were injected along the length of the EDL muscle using a 5 μ l Hamilton syringe¹⁶. The skin overlying the injection site was then closed by suturing. Between 20 and 99 days after implantation, the target EDL muscle and the neighbouring tibialis anterior and peroneus muscles were removed for analysis. GPI isoenzymes from single cryostat sections were analysed by isoelectric focusing (see Fig. 1, ref. 22). The presence of the AB heterodimeric GPI isoenzyme, lying intermediate between the AA or BB homodimers, indicates that the implanted mpc had been incorporated into host muscle fibres¹⁷.

* Number containing heterodimer/number examined.

† C57Bl/10ScSn wild type.

‡ C57Bl/10ScSn mdx/mdx.

myonuclei in mosaic muscle fibres. We did not see this dystrophin immunostaining in non-injected contralateral control muscles, or in muscles that had been injected with normal mpc but contained no donor or heterodimer GPI.

We injected C57Bl/10-mdx-derived mpc into mdx/nude hosts to control for the possibility that the injection procedure itself might have induced dystrophin expression in mdx myofibres. In muscles where the presence of heterodimer GPI confirmed that the injected mdx mpc had become incorporated into the mdx host myofibres (Fig. 1f; Table 1), dystrophin was not detectable either by immunofluorescence or by immunoblotting.

The availability of anti-dystrophin antibodies permits assessment of a possible replacement therapy for an inherited myopathy in terms of the efficacy of introduction of the potentially curative gene product. We have shown that a single injection of normal mpc into a growing or regenerating mdx muscle can lead to synthesis of normally sized dystrophin in substantial amounts and correctly located in a large proportion of muscle fibres. This almost certainly reflects *de novo* synthesis of dystrophin, rather than passive carriage of this protein by injection.

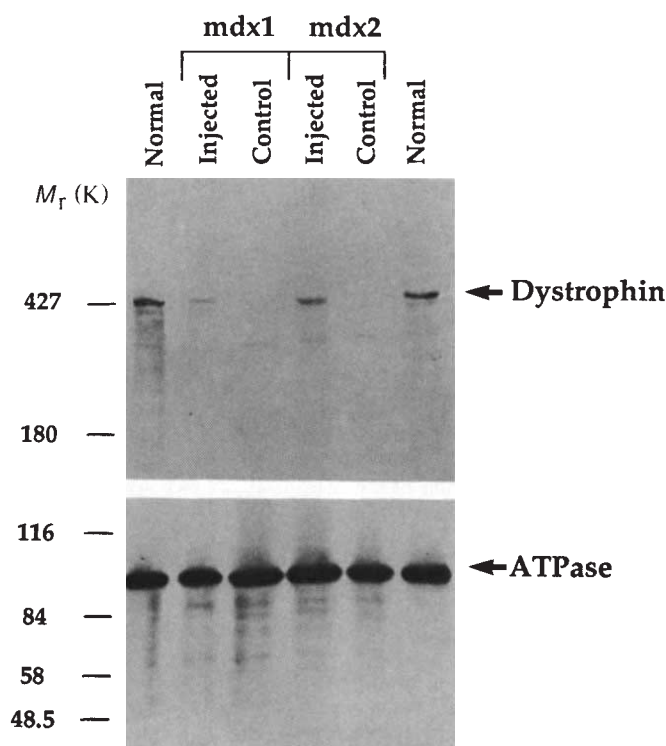


Fig. 2 Immunoblot detection of dystrophin in injected mdx muscles. Total muscle protein derived from the injected and non-injected (control) EDL muscles of two mdx mice was analysed for dystrophin content. The injected muscles have apparently normal dystrophin present at up to 30–40% of levels found in normal mouse muscle. The immunoblot, after use for dystrophin detection, was reprocessed for detection of a fast-twitch isoform of the $\text{Ca}^{2+}\text{Mg}^{2+}$ ATPase⁴¹ as a control for the muscle protein content of each lane. Relative molecular mass (M_r) calibration is shown to the left.

Methods. The portion of the EDL muscles remaining from the GPI isoenzyme and dystrophin immunofluorescence analyses were chipped out of the histological embedding medium at -20°C and processed for electrophoresis on 3.5%–12.5% gradient SDS-polyacrylamide gels and immunoblotted as previously described⁶, except that gels of 1.5 mm thickness were used and the bisacrylamide concentration in the 3.5% gel was 0.13%.

ted cells, because these cells were entirely mononucleate^{17,22,27} and dystrophin is not expressed before the formation of multinucleate myotubes^{28,29}. These findings contrast markedly with earlier work on the inherited phosphorylase kinase deficiency in the ICR/IAN mouse¹⁸, where incorporation of grafted normal mpc into host myofibres was poor and, even when successful, resulted in only a marginal increase of phosphorylase kinase above basal levels. This difference may reflect the lack of myonecrosis and regeneration in the ICR/IAN as compared with the mdx myopathy. Law has studied the murine 'dy' dystrophy^{30,31}, an autosomal recessive condition involving a major neuropathic element³²; the aetiology and pathogenesis of this disease are uncertain^{33,34}, so interpretation of the results is not straightforward. Nonetheless, the considerable functional improvement achieved by injecting dy/dy muscles with normal mpc is encouraging.

Before cell implantation can be considered as a means of therapy for DMD in man, a number of important problems must be addressed. First, it is important to establish whether the introduction of dystrophin into mdx myofibres by this means will prevent their subsequent necrosis. Second, it is difficult to see how the requirement for large numbers of human myogenic cells can be met, except by large-scale tissue culture and cell-sorting³⁵. Third, the methods used to avoid immune rejection

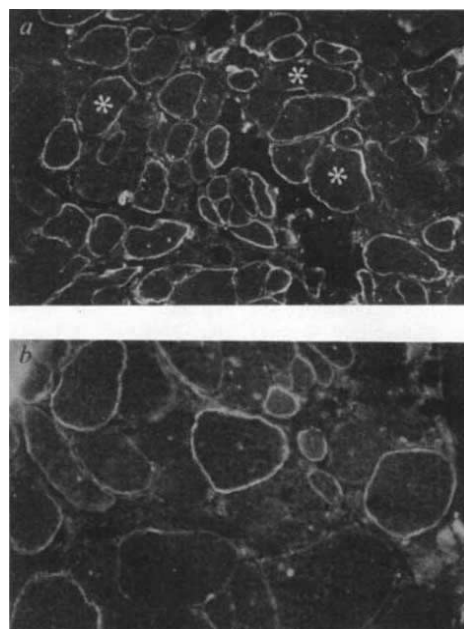


Fig. 3 Immunofluorescent visualization of dystrophin in mdx muscles injected with mpc derived from normal (C57Bl/10) donors. *a*, Muscle of M9 host mouse injected at 21 days with 1.5×10^6 mpc derived from a normal (C57Bl/10) donor and examined 21 days later. Muscle fibre profiles exhibiting the characteristic pattern of sarcolemmal dystrophin staining are found throughout the field. They are interspersed with typical mdx fibre profiles lacking this intense surface staining, and visible only by virtue of background sarcoplasmic staining. In a few fibres (marked with an asterisk), some regions of the sarcolemmal profile are weakly stained or unstained and others are brightly stained. This patchy pattern presumably reflects the restricted area of distribution of dystrophin around myonuclei derived from injected normal mpc. Stain is streptavidin-Texas Red and the final magnification is $\times 128$. *b*, Muscle of mdx/nude mouse injected at a time of muscle growth (7 days) with 4×10^6 mpc derived from a normal (C57Bl/10) donor and examined 31 days later. This shows a mixture of completely stained, partially stained and unstained muscle fibres in what appears to be an area of regeneration. It is uncertain whether these donor cells entered the muscle fibres during growth, during regeneration following the damage caused by the injection or during the regeneration that follows the onset of mdx lesions at around 21 days. The presence of some very small dystrophin-positive fibres suggests that the latter mechanism is at least partially responsible. Stain is streptavidin-Texas Red and the final magnification is $\times 200$.

Methods. Cryostat cross-sections (5–8 μm) of the muscles were thawed on to gelatin-coated slides. PBS containing 5% horse serum was used for blocking of the sections and for all reagent dilutions and washes. The primary antibody was a mixture of crude sheep-anti-mouse-dystrophin antisera directed against the dystrophin antigens of molecular weight 30,000 and 60,000 and diluted 1:500. The second antibody was a biotinylated donkey anti-sheep (Amersham), diluted 1:250, followed by a streptavidin-Texas Red (BRL) or streptavidin-fluorescein (Amersham) diluted 1:40 or 1:250 respectively.

in the present study would not be applicable to man, although the absence of class I MHC antigens on mature muscle fibres in man³⁶ and in mouse³⁷ suggests that hosts and donors may require less stringent matching for muscle grafts than for other tissues. It is also encouraging that long-term survival of muscle allografts in the mouse has been achieved with cyclosporin A^{30,38}, but the demonstration of both class I (ref. 36) and class II (ref. 39) MHC antigens on muscle fibres in some circumstances encourages caution. Fourth, there is the formidable problem of treating all the major muscle groups; as the migratory powers of mpc have not been shown to exceed a few mil-

limetres^{22,40}, injections would need to be spaced by not more than 1 cm apart. Finally, the pronounced cardiac abnormalities in DMD may not easily be addressed by the techniques described in this report.

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The mucosal vascular addressin is a tissue-specific endothelial cell adhesion molecule for circulating lymphocytes

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Tissue position-dependent or addressin-dependent expression of cell adhesion molecules has been proposed to play a part in cellular positioning in a variety of systems, for example during neural development, the metastasis of neoplasms, and the tissue-specific homing of lymphocytes. The extravasation of blood-borne lymphocytes is regulated by interactions with the endothelium of specialised venules, such as the high endothelial venules (HEV) in organized lymph nodes and mucosal lymphoid tissues¹⁻⁴. At least three separate lymphocyte-HEV recognition systems have been described, one mediating tissue-selective lymphocyte interactions with HEV in peripheral lymph nodes, another in mucosal lymphoid organs, and a third in inflamed synovium⁴⁻⁸. We have previously identified a tissue-specific 'vascular addressin' in the mouse which is selectively expressed by venules mediating lymphocyte-homing into mucosal tissues⁹. To determine whether this addressin is a specific adhesion molecule for lymphocytes, we have isolated it by monoclonal antibody-affinity chromatography and inserted it into supported phospholipid planar membranes^{10,11}. Lymphocytes bind to membranes containing the addressin, but not to phospholipid bilayers or to control glycophorin-reconstituted membranes. Only those lymphocytes and lymphoma cell lines capable of binding to mucosal HEV adhere well to the isolated addressin; peripheral lymph node HEV-specific or HEV-non-binding cell lines do not bind. Binding is blocked by anti-addressin antibody MECA-367. We conclude that the mucosal vascular addressin is a tissue-specific endothelial cell-adhesion molecule for lymphocytes, and suggest that it could regulate lymphocyte

traffic into mucosal tissues by mediating attachment of blood-borne cells to endothelium.

The mucosal vascular addressin (MAd), a glycoprotein of relative molecular mass (M_r) 58,000-66,000 (58 K-66 K) which is highly expressed by HEV in mucosal lymphoid tissues, was purified to near-homogeneity from detergent lysates of mouse mesenteric lymph-node stroma by antibody-affinity chromatography (see Fig. 1). Artificial planar lipid membranes containing the isolated MAd were prepared on quartz slides, and we assessed the ability of these membranes to support binding of normal mouse lymphocytes and of TK1, a mouse lymphoma that binds 3-5 times better than normal mouse lymphocytes to mucosal HEV in Peyer's patch frozen sections⁶. Both normal lymphocytes and TK1 cells bound better (7-10-fold) to the addressin-containing than to the control glycophorin-reconstituted membranes, or to membranes without inserted proteins (see Fig. 2 for TK1 cells). Also, TK1 cells bound roughly 5 times more efficiently than lymph node cells to the MAd-containing membranes. Under the conditions used, up to 50% of applied TK1 lymphoma cells bound. Further experiments showed that there was binding to addressin, like binding to HEV in the Stamper-Woodruff frozen-section assay², both at 7 °C and at 25 °C (not shown).

We compared binding of lymphomas that express functional homing receptors for mucosal HEV with the binding of lymphomas which recognize only peripheral lymph-node HEV, or which have no HEV-binding ability at all (Fig. 3). TK1 and TK43, T-cell lymphomas which bind to mucosal HEV in frozen sections^{6,12}, also bind well to MAd-reconstituted membranes. In contrast, the peripheral lymph-node HEV-specific B-cell line 38C13 (ref. 13), and homing receptor-negative T cell (BW5147), and B-lineage (L1-2) lymphomas^{12,13} did not bind above the background levels defined by adherence to glycophorin-reconstituted membranes. Thus the MAd selectively supports the binding of lymphoid cells which are able to recognize and adhere to mucosal HEV. The specificity of binding was further confirmed in monoclonal antibody blocking experiments (Fig. 4). Pretreatment of the membranes with anti-addressin antibody MECA-367 reduced lymphocyte binding to background levels. Control antibodies, including another anti-addressin antibody MECA-89 which does not block lymphocyte binding to HEV (ref. 9), had no significant effect.

The results indicate that the mucosal vascular addressin is a

Fig. 1 SDS-PAGE/silver stain analyses of the affinity-isolated mucosal addressin (MAd). The two independent addressin preparations used here are shown. Lanes *a*, *c* and *e* show the isolated MAd (MECA-367 column eluate), and lanes *b*, *d* and *f* the eluate from corresponding control antibody columns prepared with irrelevant class-matched monoclonal antibodies. Gels were run under reducing (*a-d*) or non-reducing (*e,f*) conditions. Molecular weight markers (in thousands) are indicated to the left.

Methods. The MAd (MECA-367 antigen) was isolated as previously described⁹, with minor modifications. Typically, minced lymphocyte-depleted stroma from ~200 6–8 week-old Balb/c mouse mesenteric lymph nodes was lysed at 4°C in 100 ml NP-40 lysis buffer (2% NP-40, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 10 µg ml⁻¹ leupeptin, 10 µg ml⁻¹ pepstatin, 1 mM phenylmethylsulphonyl fluoride, 10 µg ml⁻¹ aprotinin, 0.02% NaN₃). After clarification by centrifugation, the lysate was passed sequentially through 1 ml affinity columns of control rat IgG2a antibodies and of MECA-367 covalently bound to Sepharose-4B (Pharmacia, 5 mg ml⁻¹). Columns were washed extensively in NP-40 wash buffer (0.1% NP-40, 50 mM Tris HCl, pH 8.0, 500 mM NaCl, 5 mM EDTA, 0.02% NaN₃) and then β-octyl glucoside (β-OG) wash buffer (50 mM β-OG, 50 mM Tris HCl, pH 8.0, 500 mM NaCl, 5 mM EDTA, 0.02% NaN₃). Bound material was eluted with β-OG elution buffer (0.2 M acetic acid, 50 mM β-OG, 500 mM NaCl, 0.02% NaN₃); 400 µl fractions were collected and neutralized with 200 µl 1 mM Tris HCl, pH 8.0. More than 90% of the MAd was present in the second and third fractions, and only these were used for preparation of membranes. Conventional procedures for SDS-PAGE with silver-staining were used to characterize the affinity-isolated MAd. The MAd migrates as a 58–66 K band under non-reducing conditions. The size and occurrence of minor background bands was variable and did not correlate with the lymphocyte-binding capability of isolated MAd.

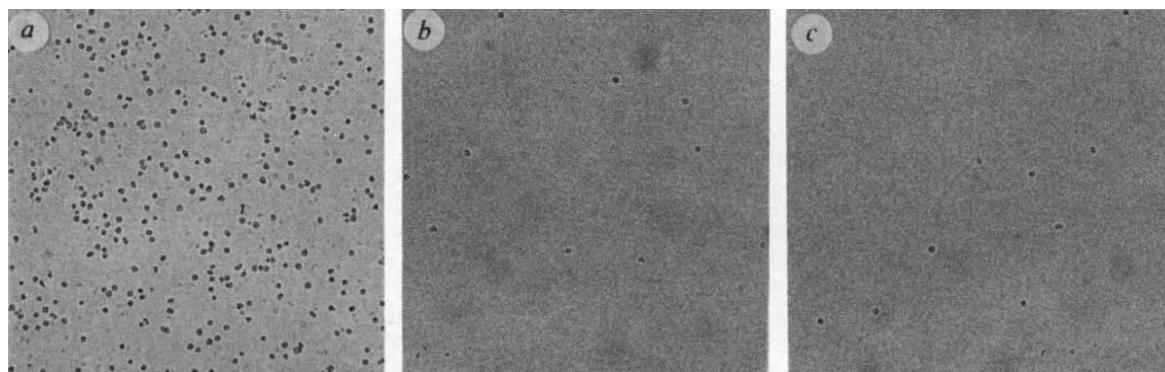
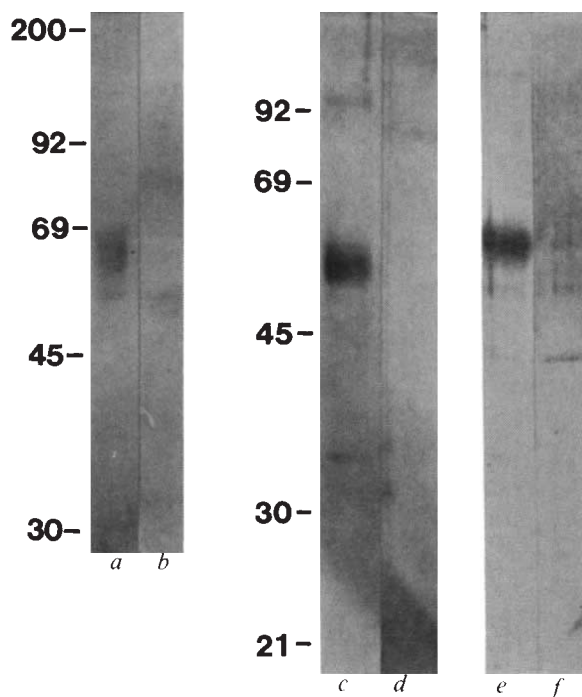


Fig. 2 Binding of TK1 lymphoma cells to membranes containing mucosal addressin (*a*), glycophorin (*b*), or no added protein (control, *c*).

Methods. Formation and reconstitution of supported planar membranes. A stock solution of saturated dipalmitoyl phosphatidylcholine (16:0) and dilinoleoyl phosphatidylcholine (18:2—an unsaturated synthetic L-α-lecithin) (Avanti Polar Lipids), was prepared at a 9:1 molar ratio in chloroform and combined at a 7:2 molar ratio with cholesterol. After complete evaporation of solvent under a stream of nitrogen, the lipids were dissolved in a solution of 25 mM β-OG in 0.01 M Tris/0.014 M NaCl, pH 8.3. The molar ratio of detergent to lipid in the micelle has to be at least 3:1 (the ratio usually used) to avoid the presence of particles large enough to cause visible turbidity. The solution was bath-sonicated to clarity, passed through a 0.2 µm-pore filter, and then diluted into a solution of the same buffer containing addressin, glycophorin, or no added protein. Reconstitution was achieved by adding 100 µl lipid solution to 1200 µl protein solution containing isolated addressin from 150–200 mice (~5 µg) or 3–5 µg glycophorin type A (Sigma). Vesicles are formed by dialysis against four 1-litre changes of Ca²⁺/Mg²⁺-free PBS over 72 h at 4°C; during prolonged dialysis unilamellar vesicles are formed. Vesicles were applied to a sucrose gradient (5–45% sucrose in PBS) and centrifuged at 80,000 g for 40 h. The vesicles were recovered as a sharp flotation band. After suspension in PBS and pelleting (100,000 g, 1 h), vesicles were dialysed overnight against serum-free RPMI and then applied to quartz slides to generate supported membrane bilayers^{11,10}. The planar membranes were washed extensively with PBS, with 0.2% fetal calf serum in PBS, and then with medium (5% fetal calf serum, 10 mM HEPES in RPMI). About 40 µl cells at 3 × 10⁷ per ml were then applied to each supported membrane. This resulted in roughly 600 cells per 0.16 mm² (1 unit area, defined microscopically by an ocular grid). After 30 min at room temperature, unbound cells were rinsed off with medium. The number of cells bound per unit area was determined microscopically by counting several fields per membrane. At least 3 membranes were analysed per sample or treatment in each experiment. Interestingly, TK1 cells, which bind extremely well to mucosal HEV in the frozen-section assay, also bound better than small lymphocytes to the isolated MAd and remained tightly bound to the MAd-containing membranes for up to 2 h after washing. In contrast, normal lymphocytes were released from the membrane as early as 30 min after assay.

lymphocyte-binding molecule. The MAd may be a ligand for one or more of the lymphocyte surface molecules that seem to be involved in recognition of mucosal HEV^{5,7,13,14}. Its selective expression on venules in mucosal tissues, including Peyer's patches, intestinal lamina propria and lactating mammary gland, provides a unique mechanism for directing the homing of specialized lymphocyte subsets to mucosal sites. The expression

of other tissue-specific lymphocyte-binding addressins, including possibly the recently identified peripheral-node vascular addressin¹⁵, may help explain the ability of various normal and neoplastic lymphocyte subpopulations to migrate selectively to peripheral (non-mucosal) lymph nodes, to inflamed joints, or to skin for example. The description of such address-dependent cellular adhesion molecules may also be relevant to mechanisms

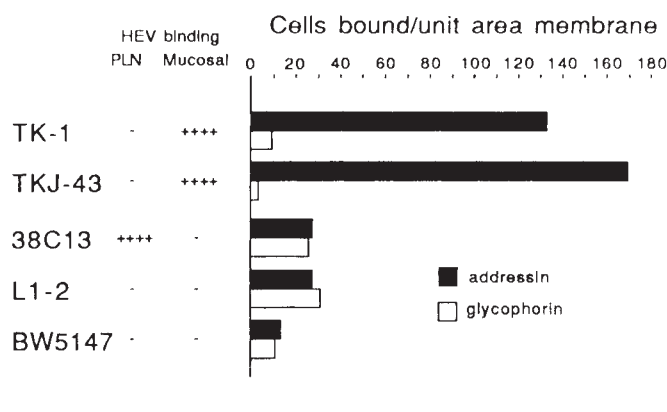


Fig. 3 Binding of lymphomas to the mucosal addressin correlates with their ability to bind to mucosal HEV.

Methods. The lymphomas used here have been described^{6,12,13}. Briefly, TK-1 and TKJ-43 are AKR/Cum and AKR/J thymic lymphomas respectively; these lymphomas were passaged *in vivo*, frozen in liquid nitrogen from recipient lymph nodes, and used immediately after thawing. 38C13, a C3H/eb B-lineage lymphoma¹⁶, was obtained directly from the lymph nodes of adoptive recipients. L1-2, a C57L pre B cell line, and BW5147, an AKRT cell lymphoma, were grown *in vitro*. The HEV-binding characteristics of these lymphomas have been described^{6,12,13} and are summarized in the figure. Because the HEV-binding of cell lines can vary, however, these binding properties were confirmed by assaying the particular lymphoma preparations used here in the *in vitro* frozen-section assay^{2,6,12}. As reported, TK1 and TKJ43 bound substantially better than reference normal lymphocytes to Peyer's patch HEV, 38C13 bound better than normal lymphocytes to peripheral lymph node HEV, and L1-2 and BW5147 were non-binders. Lymphoma binding to MAD- or glycophorin-containing membranes was as in Fig. 2. The data represent the mean results of 1-3 experiments performed on different days.

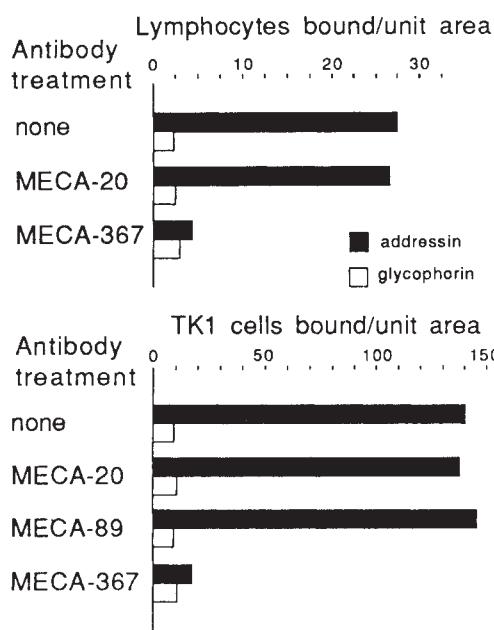


Fig. 4 Lymphocyte binding to MAD is inhibited by antibody MECA-367 but not by control antibodies.

Methods. Binding of lymphocytes or TK1 cells to the membranes was carried out as in Fig. 2, except that the membranes were preincubated with 50 $\mu\text{g ml}^{-1}$ MECA-367, MECA-89 or class-matched control antibody MECA-20 (ref. 17) (against an irrelevant endothelial cell antigen) in RPMI, 10 mM HEPES for 15 min, and then rinsed before application of sample cells. The bars represent pooled data from independent experiments with similar results, except for the binding of TK1 cells to untreated or MECA-89-treated glycophorin-reconstituted membranes, which was assayed only once. Comparable background-level binding was observed on untreated or MECA-89-treated control membranes lacking inserted protein (not shown). One of the two experiments examining TK1 binding to untreated membranes is shared with Fig. 3.

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Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures

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determining cell positioning or cell targeting in other systems, for example in the development of specific neuronal connections during neurogenesis, or the migration of differentiating embryonic cell populations during morphogenesis.

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The receptors found on most T lymphocytes bind to antigen presented on major histocompatibility complex proteins and consist of dimers of α - and β -polypeptides associated with the invariant CD3 complex^{1,2}. A fully competent immune system requires a diverse array of T-cell antigen receptors (TCRs) with different specificities. This diversity is generated by rearrangement of TCR α - and β -chain gene segments within the thymus^{3,4} where the receptors are first expressed. Any cells carrying self-reactive receptors must be eliminated, suppressed or inactivated so that destructive autoimmunity is avoided. Recently, compelling evidence has shown that one process involved in producing such self-tolerance is clonal deletion of autoreactive cells within the thymus by an

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as-yet-undefined mechanism⁵⁻⁸. Here we show that engaging the CD3/TCR complex of immature mouse thymocytes with anti-CD3 antibodies produces DNA degradation and cell death through the endogenous pathway of apoptosis. Activation of this process in immature T cells by the binding of the TCR to self-antigens may therefore be the mechanism which produces clonal deletion and consequently self-tolerance.

Anti-CD3 antibodies induce mature T cells to proliferate⁹, but immature thymocytes, expressing low levels of surface CD3, do not proliferate in response to these antibodies^{10,11}. But anti-CD3 antibodies do elicit a transitory increase in cytoplasmic calcium concentration in immature thymocytes, although the magnitude of the response is smaller than that of mature cells¹¹. As thymocytes can activate an endogenous suicide mechanism which can be triggered directly by a number of stimuli, including changes in intracellular calcium concentration^{12,13}, we investigated whether engaging the CD3/TCR complex with anti-CD3 antibodies could result in the activation of this mechanism. We studied this response in organ cultures of fetal mouse thymus, which provide an *in vitro* system that can support TCR gene rearrangement and expression⁴ and avoid the problem of low cell viability encountered in cultures of isolated thymocytes. The TCR-positive cells make up ~50% of the lymphocytes in such cultures (ref. 14, and our unpublished results) and are at an immature stage of development (see legend to Fig. 1).

Antibodies to mouse CD3 added for the last 18 hours of culture resulted in a substantial reduction in cell yield (~45%, see Table 1) and induced the degradation of DNA to oligonucleosomal bands which is characteristic of apoptosis^{12,13} (Fig. 1, lanes 3 and 4). Toluidine-blue stained sections (Fig. 2a) and cytopins of anti-CD3 treated organ cultures showed numerous apoptotic thymocytes. The chromatin condensation and cell shrinkage characteristic of apoptotic cells was also clearly shown by electron microscopy (Fig. 2b). The cell death and DNA degradation response of immature thymocytes was produced by both hamster anti-CD3 and rat anti-CD3 antibodies. The same response was also observed in chicken embryo thymus organ cultures treated with anti-chicken CD3 antibodies (unpublished work with M. D. Cooper). In contrast, the same rat and hamster anti-CD3 antibody supernatants strongly stimulated proliferation when incubated with mature T cells from mouse spleen (data not shown), as has been previously reported⁹. The response of T lymphocytes to incubation with anti-CD3 antibodies was therefore critically dependent on the stage of maturity of the cells: the same antibody treatment which caused splenic T cells to proliferate caused immature T cells to die. Significantly, depletion of the double positive CD4⁺CD8⁺ cells in organ cultures treated with anti-CD3 antibody accounted for most (83%) of the reduction in cell number (Table 1).

The effects of antibodies reacting with several other T-cell surface markers were analysed to investigate the possibility that apoptosis was only induced by antibodies reacting with the CD3/TCR complex. A rat anti-Thy1 antibody also produced stimulation of mature T cells (data not shown), but did not induce significant DNA degradation in immature thymocytes (Fig. 1, lane 2). Rat anti-CD4 antibody of the same subclass (IgG2b) as the rat anti-CD3 antibody also failed to induce apoptosis (Fig. 1, lane 5) despite the observation from fluorescence-activated cell sorting that the immature thymocytes bound substantially more anti-CD4 antibody than anti-CD3 antibody (data not shown). Thy-1 and CD4 are displayed by a large majority of such immature CD3⁺ cells¹⁰.

We tested the hypothesis that cells displaying surface receptors were eliminated by the anti-CD3 antibody, whereas cells displaying only cytoplasmic TCR β -chains were unaffected. We used monoclonal antibody F23.1 (ref. 15), that reacts with the products of the V β 8 gene family and therefore with some 20% of BALB/c TCRs¹⁵ and can be used to detect both surface and cytoplasmic V β 8-chain expression¹⁶. Immunostaining of anti-CD3 antibody-treated thymus organ cultures revealed that F23.1

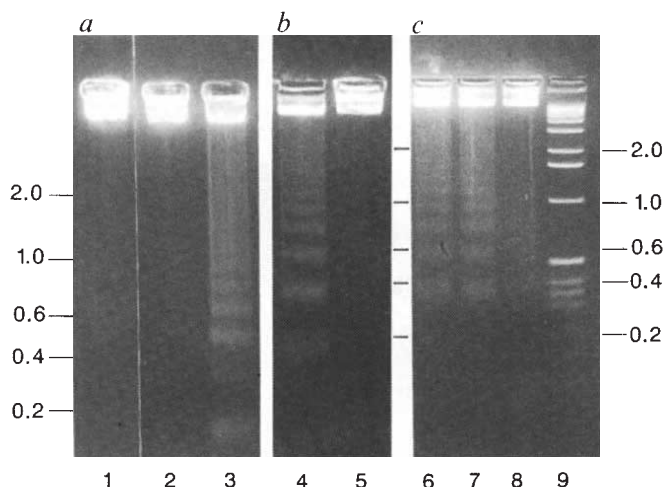


Fig. 1 Gel analysis of thymocyte DNA from cultured thymic lobes. Total DNA from 10^6 cells after treatment of cultures with (1) anti-mouse I-A^b (negative control; mouse IgG2a hybridoma 25-9-175²⁵), (2) anti-mouse Thy-1 (rat IgG2a hybridoma G7²⁴), (3) anti-mouse CD3 (hamster hybridoma 1452C11⁹), (4) anti-mouse CD3 (rat IgG2b hybridoma 29B; Portole *et al.*, personal communication), (5) anti-mouse CD4 (rat IgG2 hybridoma YTS 191.1²³), (6) ionomycin (800 ng ml⁻¹) and PMA (10 ng ml⁻¹), (7) ionomycin (800 ng ml⁻¹), (8) PMA (10 ng ml⁻¹), (9) molecular size standards (BRL). Molecular sizes in kilobases are indicated on the left for the gel in *a* and on the right for gels in *b* and *c*. Oligonucleosomal fragments appear as ladders of diffuse bands whose molecular sizes are approximately multiples of 200 base pairs.

Methods. BALB/c (I-A^d) fetal thymus lobes isolated at day 14 of gestation were allowed to develop in organ culture on the surface of nucleopore filters supported on gelatin foam sponges, as described previously¹⁶. After 7 days of culture, surface TCR expression can be detected¹⁶ and 91% of all lymphocytes present (96% of TCR-positive cells) also express J11d, which is found on immature but not on mature T cells²². On day 7 of culture, the medium was replaced with a 50:50 mixture of fresh medium and the required hybridoma supernatant, or with fresh medium containing the indicated concentrations of ionomycin and/or PMA. After further incubation overnight (~18 h), the lobes were collected, washed and teased apart in ice-cold medium with cataract knives to liberate the lymphoid component. Portions of ~ 10^6 cells were washed and pelleted as previously described²⁶, except that the centrifugation was carried out at 4 °C. Pellets were resuspended in 20 μ l 10 mM EDTA, 50 mM Tris-HCl (pH 8.0) containing 0.5% (w/v) sodium lauryl sarkosinate and 0.5 mg ml⁻¹ proteinase K, and incubated at 50 °C for one hour. 10 μ l 0.5 mg ml⁻¹ RNase A was added to each sample and incubation at 50 °C continued for a further hour. Samples were heated to 70 °C, and 10 μ l 10 mM EDTA (pH 8.0) containing 1% (w/v) low-gelling-temperature agarose, 0.25% (w/v) Bromophenol blue and 40% (w/v) sucrose was mixed with each sample before loading into the dry wells of a 2% (w/v) agarose gel containing 0.1 μ g ml⁻¹ ethidium bromide using a siliconized pipette tip. Electrophoresis was carried out in 2 mM EDTA 800 mM Tris-phosphate (pH 7.8) until the marker dye had migrated 3–4 cm.

surface-positive cells had indeed been almost completely removed (0.9%, compared with 5.0% in controls), but that the proportion of cells containing only V β 8-chains in the cytoplasm remained at 9.5 to 10.5%. It has previously been shown that incubation of thymic organ cultures with such antibodies to particular V β gene products specifically eliminates cells bearing corresponding surface receptors¹⁶.

The effects of plasma-membrane receptor stimulation of a number of cell types can be reproduced by treatment with phorbol myristate acetate (PMA) and the calcium ionophore, ionomycin^{17,18}. These components seem to act by mimicking the increase in cytoplasmic calcium concentration and protein kinase C activity which result from stimulation of cell-surface

Table 1 Phenotypes of cells in antibody-treated thymus organ cultures

Antibody present	CD4 ⁺ CD8 ⁺		CD4 ⁺ CD8 ⁻		CD4 ⁻ CD8 ⁺		CD4 ⁻ CD8 ⁻	
	Yield	%	Yield	%	Yield	%	Yield	%
None	100,800	44.6	17,600	7.8	14,900	6.6	92,600	41.0
Anti-I-A ^b	88,700	45.5	13,400	6.9	10,900	5.6	81,900	42.0
Anti-CD3	16,300	15.1	9,800	9.1	8,500	7.9	73,000	67.9

Yields are given in cells per lobe and percentages of total cells in the lobe. The overall yield of cells was reduced by 45% in anti-CD3 antibody-treated lobes relative to anti-I-A^b antibody-treated lobes (negative control) which were incubated at the same time. Fetal thymus lobes were incubated for six days before the addition of either anti-I-A^b antibody²⁵ or hamster anti-CD3 antibody⁹ and incubation for a further 18 h (as described in Fig. 1). Lobes were then teased to release cell suspensions (20 lobes per treatment) which were double-labelled for CD4 and CD8 expression by sequential incubation with monoclonal rat anti-CD4 (ref. 23), followed by goat anti-rat immunoglobulin-fluorescein. They were then washed with 1% normal rat serum to block any free anti-rat combining sites and incubated with monoclonal rat anti-CD8-biotin (Becton-Dickinson) with 1% rat serum, followed by avidin-D Rhodamine (Vector Labs). Absence of cross-reactivity was demonstrated by the presence of single positive CD4⁺ or CD8⁺ cells only in purified lymph node T cells. In all cases at least 300 cells were scored using an epi-illumination Zeiss microscope.

receptors. This combination strongly stimulates the proliferation of mature T cells¹⁰ and, as anti-CD3 antibodies induce an increase in cytoplasmic calcium in immature T cells^{10,11}, we tested the effect of PMA and ionomycin on immature thymocytes in organ culture. Figure 1c shows that ionomycin together with PMA strongly induces apoptosis in immature thymocytes, producing the characteristic DNA degradation pattern (lane 6). PMA alone had no detectable effect (lane 8), but treatment with ionomycin alone (lane 7) was apparently no less effective than when it was combined with PMA, suggesting that the increase in intracellular calcium concentration alone was sufficient to induce the cell suicide response. The DNA degradation observed in response to ionomycin alone was similar to that reported for

A23187, a calcium ionophore with somewhat different specificity, on rat thymocytes incubated *in vitro*¹².

In the studies of Kappler *et al.*⁵⁻⁷ and MacDonald *et al.*⁸, which demonstrated clonal deletion of autoreactive cells expressing receptors with affinity for I-E or MIs, it was noted that immature cortical cells with receptors of the appropriate specificity were present, but that mature medullary cells were absent. These results indicated that the deletion process normally occurs between the immature and mature phases, although a recent report showed that CD4⁺CD8⁺ immature TCR-positive cells can also be deleted in some circumstances¹⁹. Here we have shown that immature CD4⁺CD8⁺ cells are sensitive to the induction of apoptosis by anti-CD3 antibodies. The reason for this apparent discrepancy could be that autoreactive immature cortical cells in the thymus *in vivo*, although sensitive to deletion, are not normally deleted until they contact antigen on dendritic antigen-presenting cells, which are located throughout the medulla and at the cortico-medullary junction but not in the cortex. Indeed, other studies have indicated that tolerance induction within the thymus depends on contact with dendritic cells^{20,21}.

Whatever the natural interactions in the intact thymus which lead to clonal deletion, our results clearly show that engagement of the TCR complex of immature thymocytes can result in the induction of apoptosis. In view of the potency of the effect of anti-CD3 antibodies (as compared with anti-CD4 antibodies of the same isotype), it seems unlikely that the effect is mediated by opsonization or antibody-dependent cell killing involving other cells. Rather, we suggest that the effect is a direct one on developing T cells which respond to a signal (which would stimulate mature T cells) by degrading their DNA and undergoing cell suicide. Final proof of the relevance of this mechanism in clonal deletion will depend on the direct demonstration of apoptosis in developing T cells which are in contact with the antigen(s) for which they have affinity.

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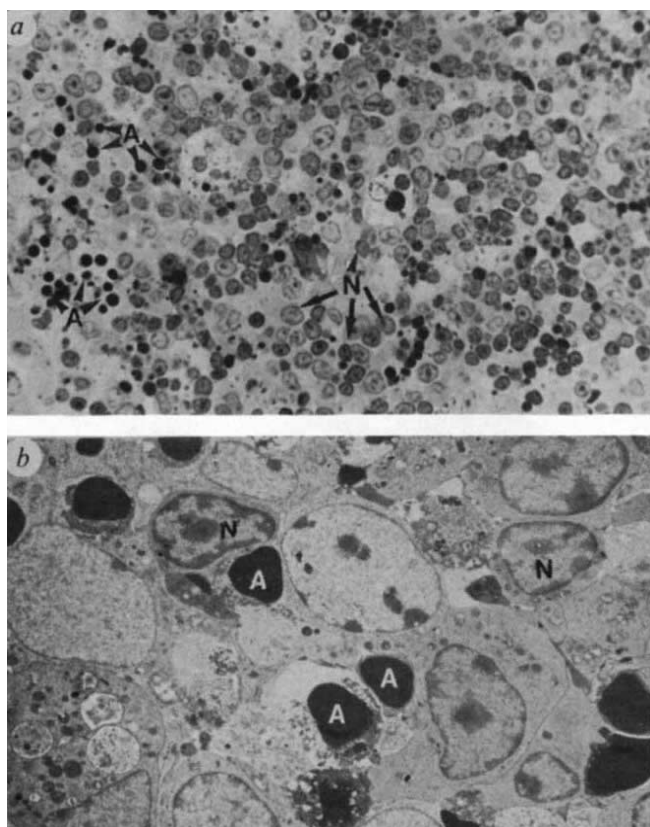


Fig. 2 Histological appearance of cells induced to undergo apoptosis in intact fetal thymus lobes after short-term exposure to anti-CD3 (as described in Fig. 1). *a*, Toluidine blue-stained 1 μ m sections. *b*, Electron micrograph taken from the same anti-CD3 treated culture as in *a*. *A* and *N* indicate representative apoptotic and normal lymphocytes respectively. Note the highly condensed state of the nuclei of the apoptotic lymphocytes.

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An Fc receptor structurally related to MHC class I antigens

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Maternal immunoglobulin G transmitted to the fetus or newborn provides humoral immunity for the first weeks of mammalian life. Fc receptors on intestinal epithelial cells of the neonatal rat (FcRn) mediate the uptake of IgG from milk. Affinity-purified FcRn is resolved by SDS-PAGE into components of relative molecular masses 45,000–53,000 (p51) and about 14,000 (p14)^{1–3}. We report the identification of the smaller component as β_2 -microglobulin. Association of β_2 -microglobulin with p51 was confirmed by cross-linking in intestinal epithelial cell brush borders. We have cloned a cDNA encoding the presumptive Fc-binding subunit, p51, and its predicted primary structure has three extracellular domains

Fig. 2 Northern blot of RNA from neonatal and adult rat tissues, probed with a 925-bp cDNA from a clone immunoselected with antiserum to the FcRn large subunit, p51. Nucleotide identities of 50–55% between p51 and some MHC class I sequences may explain the detection of hybridizing mRNAs in tissues that do not transport IgG. Lane 1, neonatal proximal small intestine; 2, neonatal distal small intestine; 3, adult small intestine; 4, adult brain; 5, adult heart; 6, adult kidney; 7, adult liver; 8, adult pancreas; 9, adult spleen. **Methods.** RNAs (25 μ g total RNA, except adult small intestine: 1 μ g poly[A]⁺RNA) were fractionated by electrophoresis in 1.5% agarose/formaldehyde gels and transferred to nylon membranes (Gene Screen Plus, NEN). Hybridization was in 5 $\times$ SSC, 50% formamide at 42 °C with a [³²P]DNA probe primed with random hexanucleotides from the 925-bp cDNA (cloning methods as in Fig. 3 legend). The membrane was washed in 0.1 $\times$ SSC at 50 °C, air-dried and exposed at –70 °C to pre-flashed Kodak XAR 5 with an intensifying screen for 8 h (top) and 36 h (bottom).

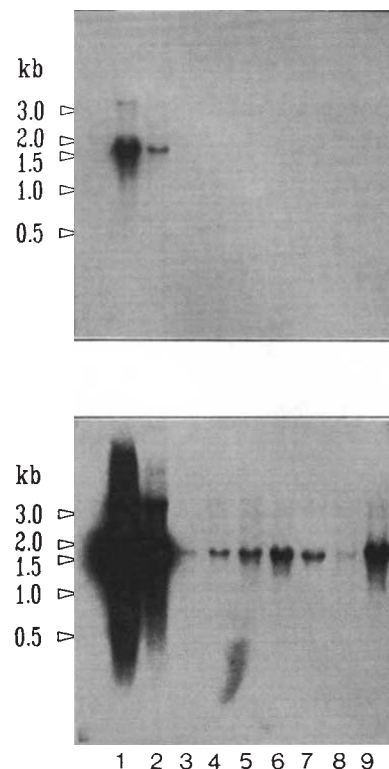
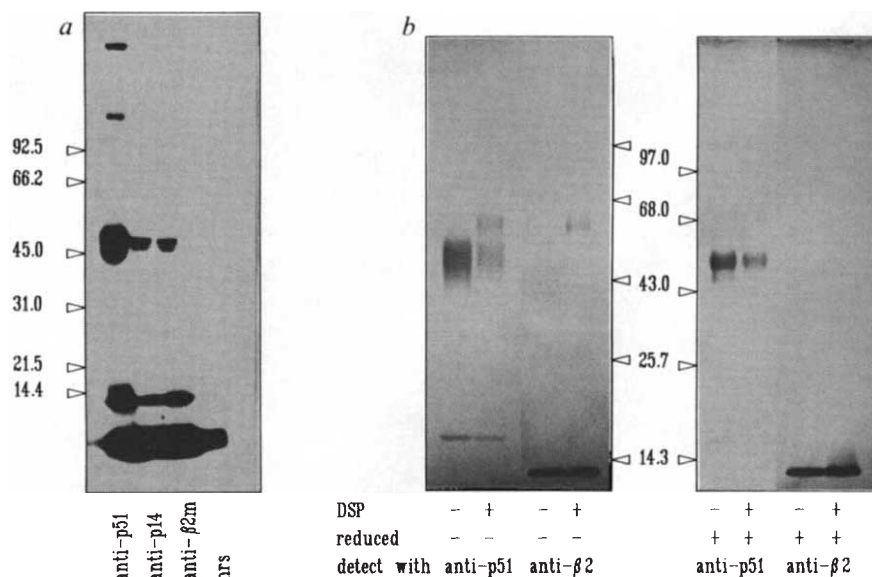


Fig. 1 *a*, Radioiodinated FcRn immunoprecipitated with antisera to p51, p14 and β_2 m, analysed by SDS-PAGE. *b*, Immunoblot analysis of FcRn prepared after crosslinking of brush border proteins. Relative molecular masses are shown in thousands.

Methods. *a*, We isolated brush borders from epithelial cells of the proximal third of the small intestine of 11-day-old Wistar rats (Charles River). Membrane proteins were solubilized in 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphate (CHAPS) in 50 mM sodium phosphate, pH 6.5, and cycled over an affinity matrix of human or rat IgG coupled to agarose. The physiological pH-dependence of IgG binding²⁷ allowed us to elute Fc_vR by shifting the pH from 6.5 to 8.0 (ref. 2). Affinity-purified FcRn was labelled with ¹²⁵I-labelled Bolton & Hunter reagent. We raised antisera in New Zealand White rabbits against the putative receptor subunits, using gel slices containing p51 or p14 cut out after SDS-PAGE as immunogen. Aliquots of [¹²⁵I]-FcRn were incubated with rabbit antisera to p51, p14 or human β_2 m or with normal rabbit serum (nrs), overnight at 4 °C in PBS/1 mg ml^{–1} CHAPS/1 mg ml^{–1} ovalbumin, pH 7.4. At this pH IgG does not bind its receptor through Fc. Protein A-Sepharose was added to precipitate immune complexes. The beads were washed in PBS/1 mg ml^{–1} CHAPS. Bound proteins were eluted by boiling in gel sample buffer containing 5% SDS and 5% 2-mercaptoethanol, then electrophoresed on 7–14% acrylamide gradient gels. The gels were dried and exposed at –70 °C to pre-flashed Kodak XAR 5 for 24–48 h with intensifying screens. *b*, Brush borders were incubated in suspension with the thiol-cleavable crosslinking reagent dithiobis(succinimidyl propionate) (DSP, 1 mM from a 100 $\times$ stock in dimethylformamide) for 4 h at 4 °C. 50 mM ethanolamine, pH 8.0, was added to quench the reaction. Control samples were prepared by adding dimethylformamide alone. The brush borders were collected by centrifugation. Membrane proteins were extracted and affinity chromatography was done as before. Crosslinked and control samples were analysed by electrophoresis under reducing and non-reducing conditions. We electroblotted the separated proteins onto nitrocellulose and probed the filters with anti-p51 or anti-human β_2 m. Alkaline phosphatase-conjugated goat anti-rabbit IgG was used as a second layer and developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazole.




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1 TCAGTTCTGTAATTAATTAACCTAACGTGGATCAAATGAGAAGGTGAAAGTTCACACAGGAGCACTCCTGTCGTCTTGACTGGGTCTCCATCCCACCATCCAGTGCCTGGTCTACGAAG 120
121 AGTCCACAGGACCTTGTGAAGATCAACAAGCGGGGTCCAGAGAGTACGTGTGCTTCCATCCGGGTGCGCTGTACAGGATGGGGATGTCACAGCCGGGGTCTCTCCAGCCTC 240
-22 MetGlyMetSerGlnProGlyValLeuLeuSerLeu -11
241 TTATTGGTCTCTGCTCAGACCTGGGGAGCGGAGCCCGTCTCCACTGATGTATCAITCTTGACGTGTGTCTGACTTATCAACGGGGCTTCCCTCTTTCTGGGCCAGGGCTGGCTG 360
-10 LeuLeuValLeuLeuProGlnThrTrpGlyAlaGluProArgLeuProLeuMetTyrHisLeuAlaAlaValSerAspLeuSerThrGlyLeuProSerPheTrpAlaThrGlyTrpLeu 30
-1 +1
361 GGTGCTCAGCAATATCTGACCTACAACAACCTGCGGAGGAGGCTGACCCCTGTGGGGCTGGATATGGGAAAACAGGTGTCTTGGTATTGGGAGAAGGAGACCAGGATCTGAAAAGC 480
31 GlyAlaGlnGlnTyrLeuThrTyrAsnAsnLeuArgGlnGluAlaAspProCysGlyAlaTrpIleTrpGluAsnGlnValSerTrpTyrTrpGluLysGluThrThrAspLeuLysSer 70
481 AAAGAACAGCTCTTCTTGGAGGCCATCAGGACCTGGAGAACCAATAAATGGGACCTTCACACTGCAGGGCCTGTGGGCTGTGAACCTGGCCCTGATAATTCTTCATTGCCACGGCT 600
71 LysGluGlnLeuPheLeuGluAlaIleArgThrLeuGluAsnGlnIleAsnGlyThrPheThrLeuGlnGlyLeuLeuGlyCysGluLeuAlaProAspAsnSerSerLeuProThrAla 110
---CHO---
601 GTGTTTCCCTCAATGGTGAGGACTTCATGCGGTCAACCCAAGACGGGCACTGGAGTGGGGAGTGGCCGGAGACAGATATCGTTGGTAATCTGTGGATGAAGCAACCTGAGCGGGCC 720
111 ValPheAlaLeuAsnGlyGluGluPheMetArgPheAsnProArgThrGlyAsnTrpSerGlyGluTrpProGluThrAspIleValGlyAsnLeuTrpMetLysGlnProGluAlaAla 150
---CHO---
721 AGGAAGGAGAGCGAGTTCCTGCTAACTTCTTGCTGAGCGGCTGCTAGGCCACCTGGAGAGGGGCGCTCAGAACCTGGAGTGGAAGGAGCGGCCATCTATGCGCTGAAGGCCGCTCCT 840
161 ArgLysGluSerGluPheLeuLeuThrSerCysProGluArgLeuLeuGlyHisLeuGluArgGlyArgGlnAsnLeuGluTrpLysGluProProSerMetArgLeuLysAlaArgPro 190
841 GGCAACTCTGGCTCCTCAGTACTGACCTGTGCTGCTTCTCTCTACCCGCGGAGCTCAAGTTTCGATTCTGCGCAATGGGCTAGCCTCAGGCTCTGGGAATTGCAGCACTGGTCCC 960
191 GlyAsnSerGlySerSerValLeuThrCysAlaAlaPheSerPheTyrProProGluLeuLysPheArgPheLeuArgAsnGlyLeuAlaSerGlySerGlyAsnCysSerThrGlyPro 230
---CHO---
961 AATGGTGATGGATCTTCCATGCATGGTCATTGTAGAGGTCAAACGTTGGAGATGAACACCATTACCAATGTCAAGTGGAGCATGAGGGGCTGGCCAGCCCTCTCACTGTGGACCTAGAT 1080
231 AsnGlyAspGlySerPheHisAlaTrpSerLeuLeuGluValLysArgGlyAspGluHisHisTyrGlnCysGlnValGluHisGluGlyLeuAlaGlnProLeuThrValAspLeuAsp 270
1081 TCGCCCGCAGATCTTCTGTGCTGTGGTTCGGAATCATCTTGGTTTATTGCTGGTGGTAGTGGCCATCGCAGGGGGTGTGCTGCTATGGAACAGGATGCGAAGTGGGCTGCCAGCCCA 1200
271 SerProAlaArgSerSerValProValValGlyIleIleLeuGlyLeuLeuLeuValValAlaIleAlaGlyGlyValLeuLeuTrpAsnArgMetArgSerGlyLeuProAlaPro 310
=====
1201 TGGCTTCTCTCAGTGGTGATGACTCTGGCGACCTATTGCCTGGTGGGAACCTTGCCCCGGAGGCTGAACCTCAAGGTGTAATGCCTTTCCGGCCACTTCTGTATGCCAACCCAGGCC 1320
311 TrpLeuSerLeuSerGlyAspAspSerGlyAspLeuLeuProGlyGlyAsnLeuProProGluAlaGluProGlnGlyValAsnAlaPheProAlaThrSer 344
1321 CATACCCATTGCAGCCTGTGGGGCTGTGTGACCTCCTGAACGTCTCTGAGCCTCCGAGGGAGCCCTGGGCTGGATGTCCTCCTCGTGATCCCTTTTGTGGCCTGCTTCAGTTTC 1440
1441 CCCTCTTAATGTCAATGGCTATTTCCATCTCCACATAAATTTGGGCCCAAATCTGTGTGTCATCGTTATTCTCAGGTTTCAGGCAGCCGGAAATAAATTGAACAAGTTTGAGAAAAAAA 1560
1561 AAAAAAAAAAAAAA 1573

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Fig. 3 The complete nucleotide sequence and deduced amino-acid sequence of the FcRn large subunit, p51. The potential ATG start is marked by asterisks over the initiation site consensus nucleotides. The predicted N terminus after signal cleavage is indicated by +1. Four potential sites for N-linked glycosylation (Asn-X-Ser or Asn-X-Thr), the hydrophobic membrane-spanning segment and the polyadenylation signal AATAAA in the 3' UT are underlined. Use of a more distal polyadenylation signal might give rise to the larger message detected on northern blots.

Methods. A cDNA library was made in λ gt11 from epithelial cells of the proximal small intestine of 11-day-old rats. Two clones were selected using anti-p51. Restriction fragments of both cDNAs were subcloned into m13 mp18 and mp19 vectors and sequenced. The smaller clone was identical to the 5' 925 bp of the larger, 1,038-bp, clone. An *Eco*RI-*Sac*I fragment comprising the 5' 350 bp was used as a probe to screen a neonatal rat intestinal library with inserts of >1.5 kb. Filters were hybridized in 5 $\times$ SSC, 50% formamide at 42 $^{\circ}$ C and washed in 0.1 $\times$ SSC at 60 $^{\circ}$ C. Two cDNAs were obtained which had identical nucleotide sequences their 5' and 3' ends and included the sequences of the smaller clones at their 3' ends. Both strands of one clone (1,570-bp) were sequenced with at least twofold redundancy.

and a transmembrane region which are all homologous to the corresponding domains of class I major histocompatibility complex (MHC) antigens. This is the first time a function has been assigned to an MHC antigen-related molecule.

We purified FcRn from isolated brush borders of epithelial cells from the proximal third of the small intestine of 11-day-old Wistar rats². The receptor was denatured in 6 M urea/20 mM Tris.HCl, pH 7.2, then diluted with five parts 0.1% trifluoroacetic acid and loaded onto a RP300 HPLC column (Brownlee). Fractions eluted in an acetonitrile gradient in 0.1% trifluoroacetic acid were analysed by SDS-PAGE. The p14 was eluted at ~32% and p51 at ~70% acetonitrile; both were electroblotted onto Immobilon polyvinylidene difluoride (PVDF; Millipore) and sequenced directly from membrane strips⁴. Additionally, p51 was digested with staphylococcal V8 protease in ammonium phosphate buffer, pH 7.8. Cleavage peptides were separated by SDS-PAGE and blotted onto PVDF for sequencing.

The amino-terminal 19 amino acids of p14 were found to be IQKTPQIQVYSRHPENGK and are identical to those of rat

β_2 -microglobulin (β_2 m)⁵. Figure 1a shows results of immunoprecipitation of radioiodinated FcRn with antisera raised against p51 and p14 and with anti-human β_2 m (Serotec). The p51 and p14 were co-precipitated by anti-p51, anti-p14 and anti- β_2 m. This supports the identification of p14 as β_2 m, and confirms its association in solution with p51. To look for a cell-surface association between p51 and β_2 m, we affinity-purified FcRn after crosslinking brush-border proteins *in situ* with dithiobis(succinimidyl propionate) (DSP). FcRn from these brush borders contained a species of $M_r \sim 62,000$ that was lost on reduction of DSP disulphide bonds (Fig. 1b). This band was detected by both anti-p51 and anti- β_2 m on western blots and so represents a p51/ β_2 m heterodimer. β_2 m has been shown previously to form a non-covalent heterodimer with products of the class I genes of the MHC^{6,7} and with CD1 (T6) antigens^{8,9}. Association with β_2 m stabilizes the heavy chains of the class I transplantation antigens in the conformations recognized by alloantisera¹⁰ and is, with few exceptions¹¹, obligatory for their export of the cell surface¹². The identification of p14 as β_2 m

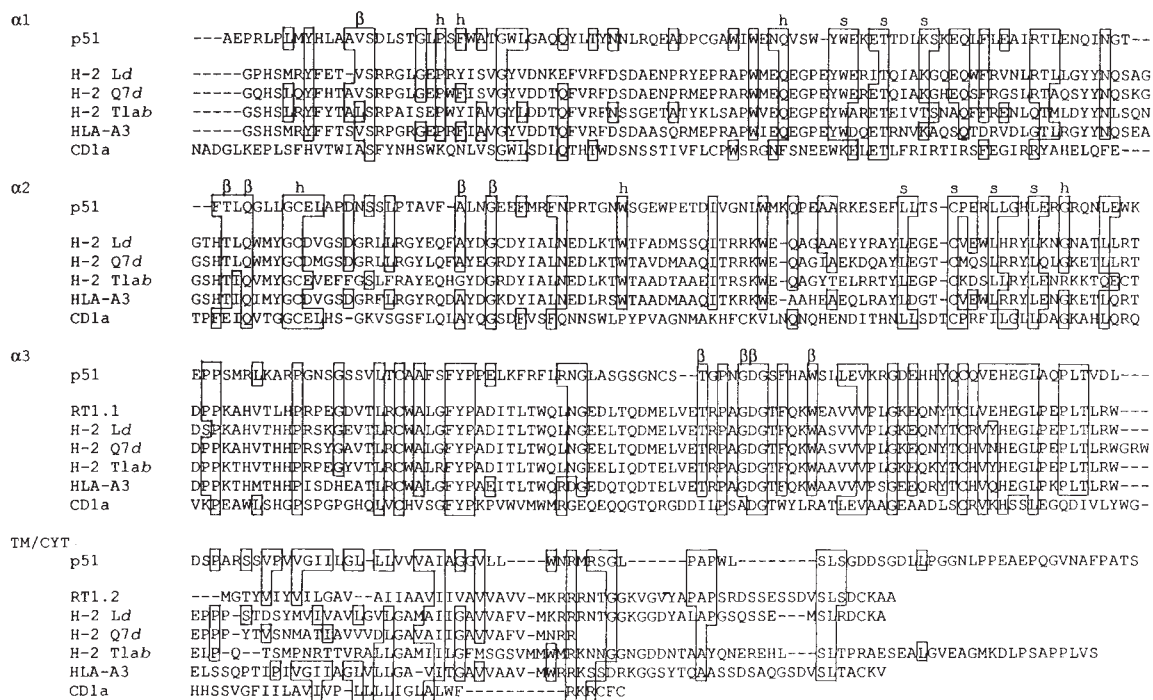


Fig. 4 Domain-by-domain comparison of the primary structures of p51, rat (RT1.1, RT1.2), mouse (H-2L^d, H-2Q7^d, H-2Tla^b), and human (HLA-A3) class I MHC heavy-chain sequences²⁸ and the thymocyte-differentiation antigen CD1a (ref. 29). Identities with the p51 sequence are boxed. Symbols above p51 residues refer to the crystallographic structure of HLA-A2¹⁶: **h** marks conserved residues (mostly in β -strands) whose side chains point toward helices; **s** marks conserved residues (in α -helices) that face β -strands; conserved residues that contact β_2 m are marked β . HLA-A2 and HLA-A3 are 95% identical in their extracellular domains; all of the marked residues are common to both.

Methods. Class I MHC sequences of each species (and subclass) that had the most identity with p51 were selected from the NBRF Protein and New databases using FASTA³⁰. The multiple alignment was generated using ALNED (Protein Information Resource staff, unpublished) and was re-gapped manually in the transmembrane and cytoplasmic (TM/CYT) domains to align stop-transfer sequences.

suggested that the larger subunit of FcRn resembles a class I MHC heavy chain.

We investigated this possibility by molecular cloning. Using the anti-p51 antiserum, we selected two clones from a λ gt11 library of duodenum and jejunum of 11-day-old rats. Both cDNAs (925 and 1,038 base pairs, bp) hybridized on northern blots with 1.7 and 3.1 kilobase (kb) mRNAs from the proximal half of the small intestine of suckling rats, which is the site of IgG transport¹³ (Fig. 2). These mRNAs are less abundant in the distal half of the small intestine, where there are fewer transporting cells¹³. Trace hybridization with an ~1.7 kb mRNA from the intestine and other tissues of adult rats was detected.

We screened a secondary library with insert >1.5 kb by hybridization at high stringency using the 5' 350 bp of one partial-length clone as a probe. One positive clone (1,570 bp) was sequenced completely (Fig. 3). An initiation AUG codon flanked by ribosome binding site consensus nucleotides¹⁴ followed ~200 bp of 5' untranslated sequence. Translation of the nucleotide sequence revealed a probable N-terminal signal sequence, a putative extracellular region with four sites of potential N-glycosylation, a single membrane-spanning domain and a 40-amino-acid cytoplasmic tail. The predicted N-terminal sequence was confirmed by the sequence AEPXLPLMYXLAAYVDL from the N terminus of HPLC-purified p51. Furthermore, predicted internal sequences matched the sequences (E163 $\uparrow$)XEFLTSXPE and (E269 $\uparrow$)XLAQPLTVDL from V8 protease fragments.

Computed alignments of p51 with class I heavy chain sequences show significant identity (ALIGN¹⁵ scores of 6–15 standard deviations) in all three extracellular domains (Fig. 4). In the X-ray crystallographic structure of HLA-A2¹⁶, domains α 1 and α 2 each comprise four β -strands followed by two or

three α -helices, and pair so that the helices are the walls and the strands the floor of a groove, proposed as the site of antigen presentation. In the α 1 and α 2 sequences of p51, helix residues that point toward the β -strands in the HLA-A2 model are notably conserved, including a region of every-fourth-residue identity in the C-terminal part of each domain (residues marked **s** in Fig. 4), corresponding to one face of each long α -helix. Few residues on the other faces of the HLA-A2 helices, which contain presumed recognition sites for the T-cell receptor and for antigen, are conserved in p51. Some β_2 m contacts are conserved, as expected (marked β in Fig. 4). The homology between p51 and the class I MHC antigens is greatest in the immunoglobulin-like domain, α 3 (35–37% identity compared with 27–30% in α 1 and 22–29% in α 2). The predicted membrane-spanning domain of p51 is related to human and (weakly) to rodent class I transmembrane regions. The cytoplasmic tails show little identity, perhaps because class I MHC molecules and p51 need different cytoplasmic sorting signals.

In the intestinal lumen of suckling rats, FcRn binds monomeric IgG. No primary structure is yet available for Fc γ RI, the macrophage receptor with high affinity for monomeric IgG, which might be predicted to resemble FcRn. Leukocyte Fc γ RII isoforms and Fc γ RIII bind immune complexes and are structurally unrelated to FcRn^{17–22}. Earlier observations however, suggest that a subset of leukocyte Fc γ Rs are associated with β_2 m: antisera to β_2 m inhibit FcR-mediated killing of opsonized cells²³ and induce cell surface rearrangement of Fc γ Rs on human peripheral lymphocytes²⁴. Cells of the Burkitt lymphoma-derived line Daudi do not make β_2 m and fail to express class I HLA antigens at their surface¹²; their capacity to bind irrelevant IgG1 monoclonal antibodies is enhanced by transfection with a cDNA encoding β_2 m²⁵. This may indicate that

some Daudi Fc γ Rs must associate with β_2m to reach the cell surface.

FcRn is much more divergent from classical class I transplantation antigens than are Qa and Tla antigens, but more closely related than CD1 molecules, which are not encoded in the MHC²⁶. No functions of Qa, Tla and CD1 have been identified, but the very different biological roles of FcRn and classical class I MHC antigens illustrate that the uses of these homologues may be diverse.

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Complete structure and expression in transfected cells of high affinity IgE receptor

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The high-affinity receptor for immunoglobulin E, Fc ϵ RI, is found exclusively on mast cells and basophils. When multivalent allergens bind to the receptor-bound IgE, the consequent aggregation of the receptors leads to the release of mediators responsible for allergic symptoms. In rodents Fc ϵ RI is a tetrameric complex of non-covalently attached subunits: one IgE-binding α subunit, one β subunit and a dimer of disulphide-linked γ subunits¹. Complementary DNA encoding the α and the β subunits has recently been isolated²⁻⁵, but expression of IgE-binding by transfected cells has not yet been achieved²⁻⁵. Here we report the cloning of cDNA for the γ subunit, and propose a model for the $\alpha\beta\gamma_2$ tetramer which accounts for many of the structural features of the receptor. The rodent receptor on the surface of COS 7 cells was expressed only when the cDNAs for all three subunits were cotransfected. Successful expression of human IgE receptors should now be possible, eventually to permit the detailed analysis of the human IgE-receptor interaction and assist the search for therapeutically effective inhibitors.

Complementary DNAs for the Fc ϵ RI γ -subunit were isolated from a λ gt11 library prepared from rat basophilic leukaemia (RBL) cells² using oligonucleotide probes. Figure 1 shows the complete nucleotide sequence of the γ cDNA, the deduced amino-acid sequence and the position in the sequence of four tryptic peptides. Analysis of the sequence suggests that there is an amino-terminal hydrophobic signal peptide of 18 residues and a putative transmembrane domain separating a short extracellular portion of five residues from an intracytoplasmic domain. As predicted by earlier studies, the N-terminal processed γ subunit contains two cysteines, no methionines and no tryptophan residue⁶. Composition analysis indicated that the γ subunit could contain one histidine residue⁶, but recent dual-

labelling studies of the receptor using ³⁵S methionine and ³H histidine indicate that no histidine was incorporated into the receptor-associated γ subunit (data not shown). As the open reading frame was derived from three independent clones, each of which predicts a histidine six residues from the C-terminal end, it is likely that the γ subunit undergoes a C-terminal processing that clips off the histidine-containing segment. Because the peptide immediately preceding this histidine was recovered (Fig. 1), the C-terminal segment must be cleaved after Lys 63. The predicted relative molecular mass of 7,139 for the fully processed γ agrees with values for the reduced γ on sodium dodecyl sulphate-urea polyacrylamide gels⁶.

Polyclonal anti-peptide antibodies to a heptamer and to a nonamer peptide of the γ subunit (Fig. 1) reacted in a western blot assay with the unreduced dimer and the reduced monomer of partially purified γ subunits. Also, both antibodies quantitatively precipitated receptor-bound ¹²⁵I-labelled IgE, either from an extract of RBL cells or from a preparation of partially purified receptors (data not shown). Taken together, these results show that the cDNAs we isolated code for the γ subunit of Fc ϵ RI.

COS-7 cells transfected with receptor cDNAs were tested for IgE-binding using an IgE-rosetting assay. Figure 2a shows IgE-binding activity expressed by cells cotransfected with the α , β and γ subunits. We now routinely achieve $5 \pm 2\%$ expression of IgE-binding by such cotransfectants. Virtually all the RBL cells used as a positive control formed rosettes (Fig. 2c). As expected⁷, the rosettes were completely inhibited by preincubation of the cells with rat IgE (Fig. 2b and d), but not with human IgE (not shown). To study the requirements for surface expression of IgE-binding activity, the cells were transfected with different combinations of the cDNAs for the three subunits. Transfection was successful for all combinations as assessed by northern blotting, but rosette-forming cells were only detected after cotransfection of the full set of the cDNAs. These results suggest that only tetrameric receptors reach the plasma membrane. This phenomenon has been observed in other systems^{8,9} and may be generally applicable to hetero-oligomeric membrane proteins.

The easy dissociability of β and γ_2 from α (ref. 10) has raised persistent uncertainty as to whether γ_2 and β should be considered subunits of Fc ϵ RI or as 'receptor-associated' proteins. We have previously argued in favour of the subunit model for Fc ϵ RI on the basis of the coordinate biosynthesis and catabolism of α , β and γ_2 (ref. 11). Our new data on transfected cells

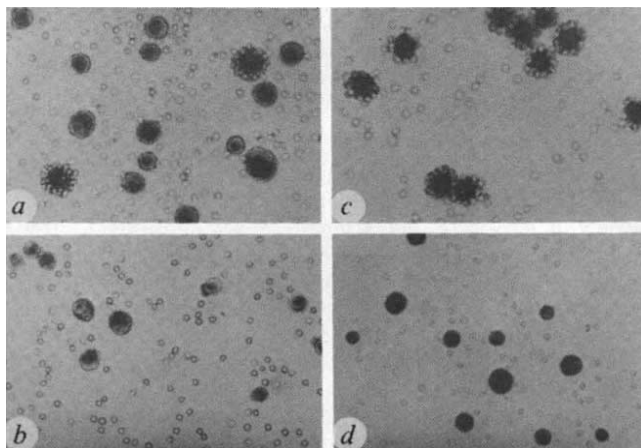
Fig. 1 Nucleotide sequence of the γ -subunit of rat Fc ϵ RI and the amino-acid sequence that it predicts. The putative transmembrane domain is underlined. Amino-acid residues are numbered starting with the first residue of the mature protein. Residues 5' to residue 1 have negative numbers and include the residues encoding a putative signal peptide, according to the criteria of Von Heijne²¹. The N-terminal and C-terminal cleavage sites are indicated by an arrow. The four tryptic peptides that were recovered and sequenced are bracketed. An asterisk denotes an ambiguous residue in the sequence of the first tryptic peptide.

Methods. Fc ϵ RI was purified by affinity chromatography using trinitrophenyl-lysine beads as described⁶. The eluate was applied to Sepharose 4B beads coupled by cyanogen bromide to monoclonal anti- β (JRK) (ref. 18). After washing the beads with 2 mM 3-((3-cholamidopropyl)dimethyl-ammonia)-1-propanesulphonate in borate-buffered saline, pH 8, the bound material was eluted at 65 °C with 0.1% sodium dodecyl sulphate, phosphate-buffered saline, pH 6.5. The subunits from Fc ϵ RI were then separated by HPLC size chromatography, the β - and γ -containing fractions recovered, reduced and alkylated and digested with trypsin². The resulting peptides were separated by HPLC-reverse phase chromatography as before². The chromatograms from the β and γ digests were compared and the non-overlapping γ peptides sequenced². Oligonucleotide probes were synthesized according to the sequences of peptide 3 (residues 41 to 47) and of peptide 4 (residues 54 to 62). Their sequences were GA⁴AA⁶TCIGA¹GCTGTCTA and AA²CA⁶GAG⁶ACITAT¹GA⁴ACI²TIAA. The methods used to screen the λ gt11 library and to purify, subclone and sequence the positive clones have been described². Peptides 3 and 4 were also synthesized using a peptide synthesizer ABI 431A. The purity of the synthetic peptides was assessed by HPLC reverse-phase chromatography, amino-acid composition and mass spectroscopy. The peptides were conjugated either to ovalbumin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester²² at a molar ratio of 5:1 or to Sepharose 4B with cyanogen bromide. Rabbits were immunized with the ovalbumin-conjugated peptides, the antisera collected and the anti-peptide antibodies purified by affinity chromatography using Sepharose 4B-conjugated peptides. The anti-peptide antibodies were tested for reactivity with the γ subunit of Fc ϵ RI by western blotting, and for their ability to immunoprecipitate ¹²⁵I-labelled IgE receptor complexes¹⁸.

AGCGCTGCAGCCCCGCCGAGG	ATG	ATC	CCA	GCG	GTG	ATC	TTG	TTC	46
	M	I	P	A	V	I	L	F	-11
TTG	CTC	CTT	TTG	GTG	GAA	GAA	GCA	GCT	91
L	L	L	L	V	E	E	A	A	5
								-1	+1
CTC	TGC	TAT	ATC	CTG	GAT	GCC	ATC	CTG	136
L	C	Y	I	L	D	A	I	L	20
CTT	ACC	CTG	CTC	TAC	TGT	CGA	CTC	AAG	181
L	A	L	L	Y	C	R	L	K	35
								{I ² }	
GAC	ATA	GCC	AGC	CGT	GAG	AAA	TCA	GAT	226
D	I	A	S	R	{E	K	S	D	50
								A	
AAC	ACC	CGG	AAC	CAG	GAG	ACA	TAT	GAG	271
N	T	R	{N	Q	E	T	Y	E	65
								T	
CCA	CCC	CAA	TAG	CTTTACAACACGTGTCTCAGCTGCATTCCTTTTCGGCTTTTA					326
P	P	Q	-						68
ATTCTCTCTCGCCCTCATGATTGACGTGGCTGTGCTACCTCCGTCTCTGGAAGTAC									385
CTGACCTTATTCGAGAACCATGCTAGGCTCTAAATCAATGTCCCATATCCACCAAG									444
ACTTACTCACTGACATTCTCTCTCGCATCCTCTTGTCTTCTCTCTCTCTCTCTCC									503
CTGATCTCTGTGCTCAATAAATGGGAAGGATTACCCCAATAAAGTGCCAGA									562
GATCAGCGTCAAAAAAAAAAAAAA									586

Fig. 2 Formation of IgE rosettes by transfected COS 7 cells and RBL cells. COS 7 cells were cotransfected with the coding portions of α , β and γ cDNAs and sensitized with mouse IgE anti-dinitrophenyl before being exposed to red cells derivatized with trinitrophenyl (panel a). As a positive control, RBL cells were similarly tested for rosette formation (panel c). The specificity of the rosetting assay was assessed by preincubating the cotransfected COS 7 cells (panel b) and RBL cells (panel d) with rat IgE (which lacks the anti-DNP activity) before the addition of the mouse anti-DNP IgE.

Methods. The 810-base pair (bp) *Eco*RI-*S*tyI restriction fragment of the α -cDNA, the 965-bp *Eco*RI-*Eco*RV restriction fragment of the β cDNA and the 300-bp *Eco*RI-*Dde*I restriction fragment of the γ cDNA were subcloned separately into the *Sma*I site of the transient expression vector pSVL (Pharmacia, Sweden). These restriction fragments individually contain the entire coding sequence of the appropriate subunit and variable portions of untranslated sequences. The only foreign sequence is the starting *Eco*RI recognition sequence that belongs to the initial linker. Cultured COS 7 monkey kidney cells were then transfected with 40 μ g DNA by the standard calcium phosphate precipitation technique²³. After 48 h the transfected cells (panels a, b), as well as RBL cells (c, d), were examined for surface expression of IgE-binding by an IgE-rosetting assay. The cells (5×10^6 cells ml⁻¹) were incubated at room temperature with (panels b, d) or without (panel a, c) 50 μ g ml⁻¹ of non-specific rat IgE for 30 min and then with 5 μ g ml⁻¹ of anti-DNP-IgE (ref. 24). The cells were then rosetted with ox red blood cells that had been modified with 2,4,6-trinitrobenzenesulphonic acid²⁵.



provide possibly the strongest evidence for $\alpha\beta\gamma_2$ being the minimal structure for Fc ϵ RI.

Our proposed model for the tetrameric receptor is shown in Fig. 3. Hydropathicity plots¹² indicate one, four, and one hydrophobic domains for the α , β and γ respectively—that is, seven transmembrane domains for the entire receptor. Members of a family of receptors interacting with G proteins that include β - and α -adrenergic receptors, muscarinic receptors and rhodopsin, also contain seven transmembrane domains¹³. Although an interaction between Fc ϵ RI and G proteins has been postulated^{14,1}, we can find no sequence similarity between Fc ϵ RI and these receptors. The topology of the α and β subunits has been discussed elsewhere^{2,3,5}, in particular the cytoplasmic localization of the C- and N-terminal portions of the β (ref. 5).

Two results are evidence for the proposed topology of the γ dimer as drawn in Fig. 3: the γ can be oxidatively iodinated on inverted vesicles but not on intact cells¹⁵, and γ becomes phosphorylated on threonine residues *in vivo*¹⁶. None of the relevant residues is present in the small presumptive extracytoplasmic segment of γ , but all are present on the presumptive cytoplasmic tail: two tyrosine and four threonine residues.

The putative extracellular and intracellular segments of the three subunits were also analysed for their relative content of basic residues¹⁷. Our model shows a good correspondence between the calculated ratios and the ratios expected on the basis of known membrane proteins¹⁷ and so independently supports the topology we propose.

Several observations were used to develop the model. The β

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Coexistence of A- and B-form DNA in a single crystal lattice

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It is well known that DNA can exist in a variety of conformations which can be interconverted by relatively mild changes in conditions. The *in vivo* conformation of DNA is usually thought to be the B form, but there is recent evidence that other conformations may be important in DNA-protein recognition. Different fragments of DNA crystallized under virtually identical conditions can form A, B or Z helices¹. A fragment that adopted an A conformation in a crystal was found in the B conformation in solution², whereas NMR spectroscopy of A-DNA films³ revealed the presence of a substantial amount of disordered B-DNA. Until now, however, a DNA fragment of a given sequence has not been crystallized in more than one global conformation^{4,5}. We report here an X-ray diffraction study of crystals of the DNA octamer dGGBrUABrUACC. In addition to a 'framework' of A-DNA, which gives discrete X-ray reflections, there are partially disordered B-DNA helices, recognized by their diffuse scattering features.

The study was carried out on single crystals of the A-DNA octamer d(GGBrUABrUACC)⁶, using high intensity X-rays

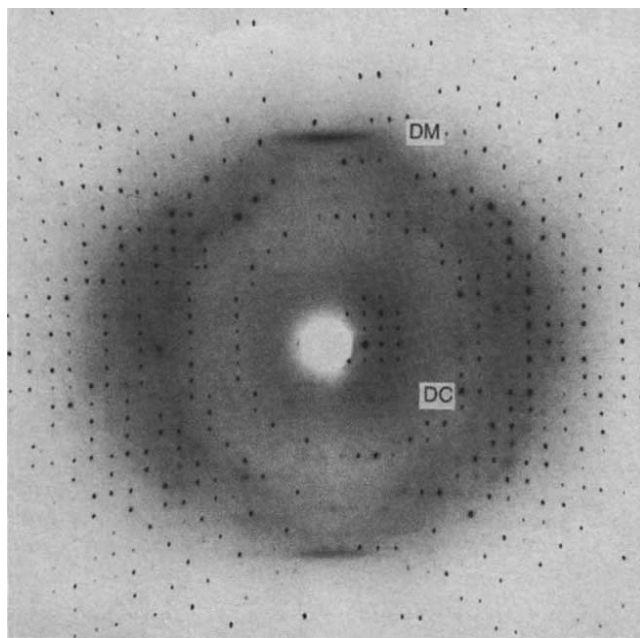


Fig. 1 One of a series of 2° oscillation photographs, from a single crystal of d(GGBrUABrUACC)⁶. The X-ray diffraction recorded on the film consists of discrete Bragg reflections and continuous diffuse rings, characteristic of a single crystal mounted in a glass capillary with mother liquor. In addition the film shows diffuse streaks, labelled DM and DC reminiscent of fibre diffraction patterns. The film was obtained with an Arndt-Wonacott camera at a crystal-to-film distance of 50 mm using synchrotron radiation of wavelength 1.42 Å. The crystal was mounted with the *c* (*c*^{*}) axis along the rotation axis (vertical on the photograph). The diffuse streaks DM and DC have ∞/m symmetry. The mean spacing of DM corresponds to 3.37 Å, close to the 3.4 Å spacing of the base pairs in B-DNA. The width of DM is $6 \times 10^{-3} \text{ Å}^{-1}$.

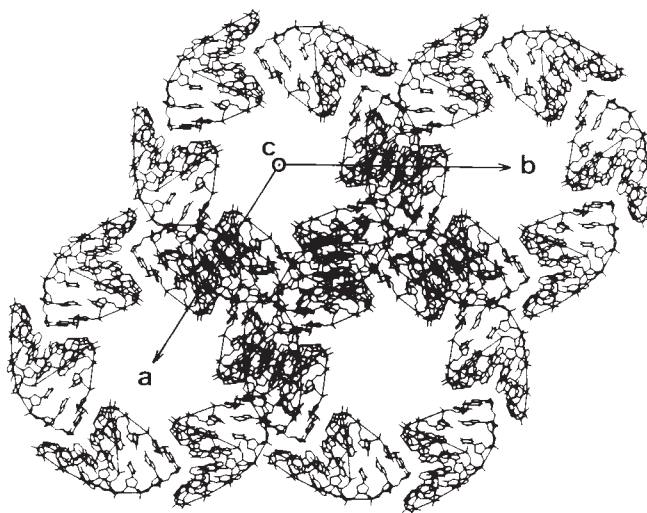


Fig. 2 Projection of the structure of d(GGBrUABrUACC) on to the (*a*, *b*) plane. The octamer crystallizes in the hexagonal space group $P6_1$ with one double helix in the asymmetric unit. The cell parameters are: $a = b = 45.05 \text{ Å}$, $c = 41.72 \text{ Å}$. The octamers adopt an A-DNA conformation with 10.9 base pairs per turn, a mean tilt angle of 18° and an average separation between base pairs of 3.4 Å. The packing can be reconstructed from the coordinates of the isomorphous octamer d(GGGCCCCC)¹⁰ deposited with the Protein Database Brookhaven National Laboratory, New York.

from the DCI storage ring at LURE. BrU is 5-bromouridine, an analogue of thymine. Figure 1 is a reproduction of one of the rotation photographs taken around the *c* axis. It shows the discrete reflexions corresponding to diffraction by the ordered lattice of A-DNA helices. It also contains two unexpected scattering features: a prominent, broad meridional streak (DM), at a *d*-spacing of 3.4 Å, and a series of narrow streaks, perpendicular to the *c*^{*} direction (DC), which are distributed in the pattern of a cross. These features are reminiscent of the helical

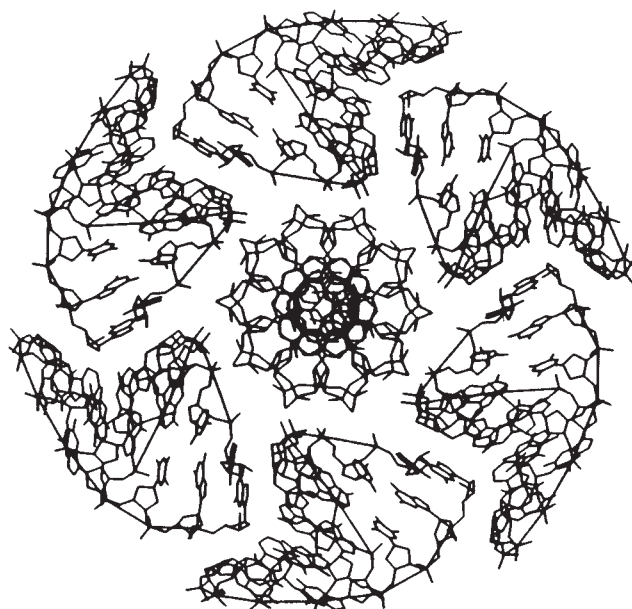


Fig. 3 Projection of the d(GGBrUABrUACC) structure showing one channel containing one occluded B-DNA octamer and the surrounding six A-DNA helices, related by the 6_1 screw axis. For clarity, a complete turn of B-DNA is used in the illustration. The steric fit is excellent when the B-DNA is correctly positioned with respect to the wall of the channel formed by the A-DNA octamers; there are no unreasonable intermolecular contacts.

Fig. 4 Comparison between an experimental film and simulations performed using A and B conformations for the occluded molecules. *a*, The experimental film is shown; *b*, *c*, the two simulations (*b* for the A-form and *c* for the B-form). These simulations were based on a combination of three types of disorder. (i) Substitution disorder described by equation (1) (see text), to account for the positions of the streaks. A periodicity of $2c$ (83.4 Å) and a fault probability of $P < 0.075$ (deduced from a comparison between the width of the calculated intensity I_D and the observed width of the streaks) was used. The observed widths approximate to those of the Bragg reflexions. (ii) Disorder of the 'second kind'⁹ to allow for the broadening of DM. This disorder is characterized by the disappearance of long range order, consequent on the distance between two crystallographic nodes ($2r$) not being perfectly defined. Equation (1) becomes:

$$I_D = \frac{1}{4}(f_x - f_y)^2 \frac{1 - (\alpha\rho)^2}{1 - 2\alpha\rho \cos(2\pi\mathbf{S} \cdot \mathbf{c}) + (\alpha\rho)^2} \quad (2)$$

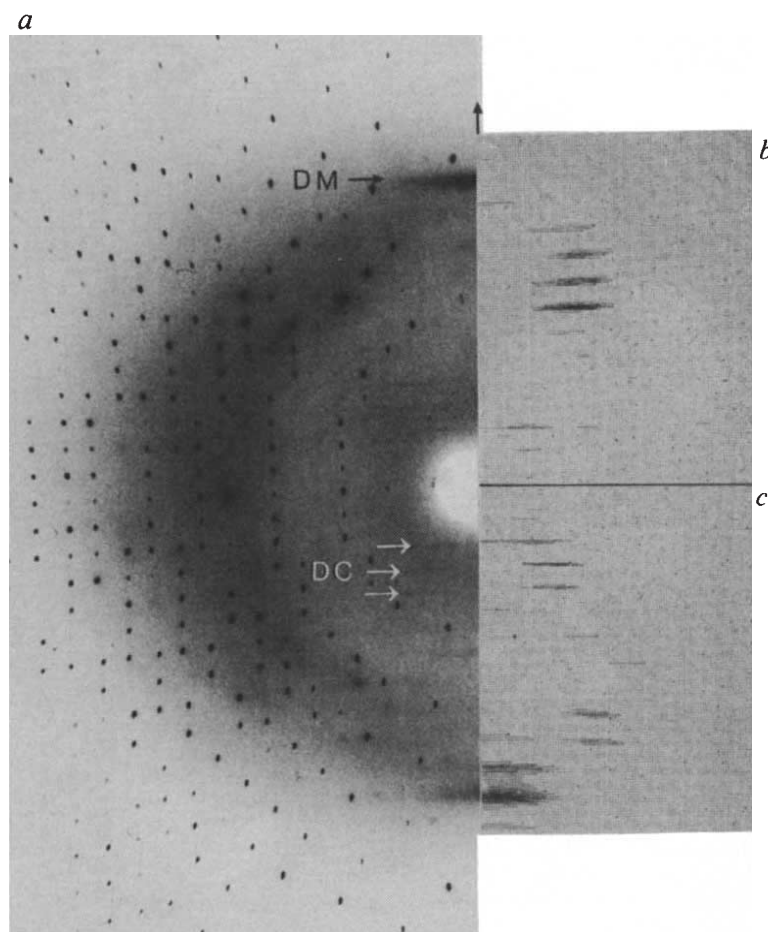
with $\rho = \exp(-2\pi^2 S^2 \Delta^2)$, where Δ^2 is the distribution variance of the distance between two nodes (assuming a gaussian distribution). Equation (2) leads to satisfactory thicknesses of both DM and DC assuming $\Delta^2 \leq 0.65 \text{ Å}^2$. (iii) Displacement disorder¹¹ to account for the relative intensities of DM and DC. In this type of disorder individual octamers or clusters of octamers have global molecular translations $\mathbf{u}$ parallel to $\mathbf{c}$ inside one channel and without correlation between channels. The effect is to multiply I_D by the expression:

$$\{1 - \exp(-4\pi^2 S^2 \langle u^2 \rangle)\} \quad (3)$$

Values of $\langle u^2 \rangle < 0.5 \text{ Å}^2$ lead to correct relative intensities. Combining these three types of disorder leads to:

$$I_D = \frac{1}{4}|F|^2 \frac{1 - (\alpha\rho)^2}{1 - 2\alpha\rho \cos(2\pi\mathbf{S} \cdot \mathbf{c}) + (\alpha\rho)^2} \times \{1 - \exp(-4\pi^2 S^2 \langle u^2 \rangle)\} \quad (4)$$

used in the simulation. In Fig. 4*b*, the scattering factor F is calculated from standard A-DNA fibre coordinates for one octamer¹². The correct base sequence was used and the methyl groups on the thymines were replaced by bromine. Solvent molecules are not included. The film intensity is simulated by a total of 17,800 pixels. The intensity is corrected for beam collimation and polarization effects¹³. To simulate an orientational disorder around the helix axis, ten simulations at 36° intervals were summed. The parameters used were $\alpha = -0.85$, $\Delta^2 = 0.65 \text{ Å}^2$ and $\langle u^2 \rangle = 0.5 \text{ Å}^2$. Note that the period in the substitution disorder was chosen as 41.0 Å instead of 41.7 Å ($=c$) as DM does not correspond exactly to a half-integer l index. The simulation does not match the experimental film very well. The meridional streak, DM is absent. There are three intense bands away from the c^* axis which result from the steep tilt of the bases in the A conformation. *c*, Simulation performed with the same conditions as in *b*, but using B-DNA fibre coordinates¹². The agreement with the experimental film (*a*) is good for both DC and DM. The scattering features near DM were found to be very sensitive to small changes in the B-DNA conformation whereas DM remains prominent.



cross and meridional reflexions observed on DNA fibre diffraction photographs⁷.

In fibre patterns the appearance of a cross (DC) is indicative of a helical structure⁸. The stacking of the base pairs along the helix axis gives rise to the meridional reflexion whose position and intensity is related to the base-pair separation and tilt angle. The separation is 3.4 Å in both the B and A conformations but the tilt angle is about 2° in the B and about 18° in the A form⁷. B-DNA generally appears under conditions of high water activity, but if this activity is reduced (on addition of salt or organic solvents), transformation to A-DNA may occur.

By analogy with fibre diffraction, the diffuse cross DC, on our photographs (Fig. 1) arises from a helical structure, whereas the meridional streak (DM) indicates a stacking of planes at a period of $\sim 3.4 \text{ Å}$. The A-DNA helices in the single crystal structure are, as shown in Fig. 2, inclined at 60° to the sixfold screw axis ($\mathbf{c}$) and interact by minor-groove contacts to form infinite spirals. Adjacent spirals interleave and enclose continuous channels parallel to the $\mathbf{c}$ axis. Clearly this ordered framework cannot be the origin of the extra scattering as it contains no helical features parallel to $\mathbf{c}$ with a repeat distance of 3.4 Å. The diameter of the channels however, (22 Å) (Fig. 3) is large enough to accommodate additional DNA molecules.

The crystal structure would thus be similar to a clathrate structure with A-DNA helices as the host molecules and occluded DNA helices as guests. These guests are likely to have the same composition as the hosts, given the high purity of our sample.

What is the stacking mode of the molecules within a given channel? If the guest molecules were to form a continuous helical DNA fibre, then we would observe the classical X-ray pattern of a cross with streaks located at n/p where n is an integer and p is the pitch of the helix. The value of p is generally between 28 Å and 34 Å depending on the global conformation. In our photographs, however, the streaks are not located at integer values of n and the value of p is not in the normal range. The streaks lie on positions $(n + 1/2) \times 1/c$ where c is 41.7 Å, the length of the crystallographic $\mathbf{c}$ axis. We can account for this observation by replacing the model of a regular extended helical structure by one in which the guest molecules are arranged in the channels in a specific stacking mode characterized by a substitution disorder. This disorder corresponds to a perturbation of the electron density which is not perfectly periodic.

The effect of substitution disorders, common in metallic alloys, is discussed elsewhere⁹. In the case of a one-dimensional row with equi-spaced atoms X and Y, separated by a distance

r , the deviation from the ordered alloy XYXYXY..., with periodicity $2r$, gives rise to diffuse scattering. The intensity I_D is given by the expression:

$$I_D = \frac{1}{4}(f_x - f_y)^2 \frac{1 - \alpha^2}{1 - 2\alpha \cos(2\pi \mathbf{S} \cdot \mathbf{r}) + \alpha^2} \quad (1)$$

where $\alpha = 2P - 1$, P being the probability of finding two consecutive atoms of the same type, f_x and f_y are the atomic scattering factors, and $\mathbf{S}$ is the scattering vector. For small values of P , I_D is located along diffuse planes perpendicular to $\mathbf{r}$, at positions $(n + 1/2) \times 1/r$. The width of the streaks is related to P , but is independent of $\mathbf{S}$. We can successfully apply this type of disorder to our case by assuming that the octamers are packed within the channels with the periodicity $2c$, (where $|c|$ is 41.7 Å) (Fig. 2).

Within a distance of 83.4 Å ($2|c|$) it is possible to accommodate three continuous octamer helices. In our model therefore, the $2c$ periodicity can arise from a distribution of XYXYXY... where X is one octamer and Y is nothing or X is two octamers and Y is nothing. The scattering factor f_x is then equal to the molecular scattering factor F of either one or two octamers.

Disorder of this type accounts for the localization of DC and would also give rise to a meridional streak at 3.4 Å corresponding to the reinforcement of the molecular structure factor by the base stacking. However, the meridional streak would be narrow as the width of the diffuse streaks is independent of the scattering vector $\mathbf{S}$. We can account for the observed width of the meridional streak (Fig. 1) by either of two models. In the first, the substitution disorder is combined with a 'second kind' disorder⁹. This disorder is characterized by the disappearance of the long range order consequent on the distance between two neighbouring crystallographic nodes ($2r$) not being perfectly defined, but given by a probability distribution. The effect is to broaden the scattering features as $\mathbf{S}$ increases. Finally the relative intensities can be reproduced by assuming a displacement disorder.

To account for the observed experimental features computer simulations were carried out using this model and either the A or B conformation. The results are shown in Fig. 4b and c. It is clear that only the B-DNA model (Fig. 4c) fits the experimental film.

The alternative interpretation of our experimental observations is to regard them as a superposition of diffuse scattering and Bragg scattering. The diffuse scattering arises from the previously described substitution disorder. The observed width of DM could be accounted for by Bragg reflexions from bases stacked perpendicular to c at about 3.4 Å separation. The Bragg reflexions would superimpose onto the diffuse streaks near DM. Only the B conformation could fit this model. Four or five regularly stacked octamers dispersed between regions of substitution disorder, but not forming a continuous helix, could produce the observed width.

It is difficult to estimate the proportion of occluded molecules. Nevertheless, as the diffuse features are comparable in intensity to the Bragg reflexions from the ordered lattice, it is probable that the occluded molecules occupy at least 10% of the space in the channels. In view of the disorder it is not surprising that the occluded octamers were not seen in the electron density maps^{5,6}.

The diffuse reflexions are prominent on photographs from d(GGBrUABrUACC), the most ordered of all the A-type octamer crystals we have examined. It is possible that the presence of DNA in the channels stabilized the crystal structure by decreasing the mobility of the solvent. The meridional streak, but not the diffuse cross, was observed on diffraction photographs from other sequences forming isomorphous A-type crystals, both by us, and by others. These observations support the model involving Bragg scattering. They suggest that small amounts of B-DNA occur in crystals of other sequences but are confined to short stacked regions only.

Our results can be summarized as follows. The diffuse scattering features DC and DM observed on the single crystal photographs can be explained by the presence of occluded B-DNA octamers within the channels of the crystalline matrix of A-form octamers. Details can be modelled either by assuming that the location of the occluded octamers is characterized by a combination of different types of disorder or that regions of substitution disorder are interspersed with short stretches of regularly stacked and rotationally disordered octamers. Whatever the exact disposition of the occluded octamers, there can be no doubt that they adopt the B and not the A conformation. The study presented shows clearly that under certain conditions of crystallization the A and B conformations of the same DNA fragment can co-exist in the highly hydrated environment of the crystal lattice.

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